No. 74
M
A-H
TR-1

VOLUME XX JANUARY 1959

THE JOHN CRERAR LIBRARY
JAN 5 - 1959

American Journal of

Veterinary RESEARCH

In this issue . . .

- Ascaris Suum in Pigs 7
- Enteroviruses in Cattle 27
- Feline Infectious Anemia 33
- Parasite Therapy in the Horse 69
- Pneumonia in Lambs 78
- Titration of Newcastle Disease Virus 116

CONTENTS ARE ON PAGES 2, 4 AND 5

XUM

Classified Advertisements Accepted in Research Journal

The *American Journal of Veterinary Research* accepts classified advertisements from graduate veterinarians seeking veterinary technological positions with colleges, universities, and other institutions such as industrial and commercial research laboratories.

Also, classified advertisements are accepted from institutions and other organizations which may be seeking veterinary bacteriologists, parasitologists, pathologists, or other veterinary technological personnel.

This service is intended to facilitate the placement or finding of personnel with specialized training in veterinary medical science.

The rate for these classified advertisements is $4.50 for the first 25 words and 15 cents for each additional word. No charge is made for use of a box number.

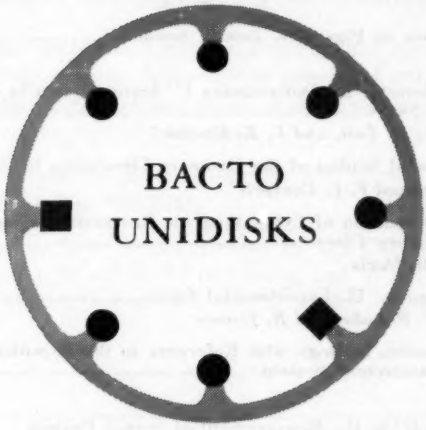
The closing date for classified advertisements in this *Journal* (a bimonthly) is the 20th of the month two months preceding date of issue.

Printed in the U.S.A.

XUM

DIFCO

for MICROBIAL SENSITIVITIES . . .



Now--at one time--a single unit permits rapid, reliable determination of microbial sensitivity to 8 different therapeutic agents

Bacto-Unidisks are prepared in three concentrations (low, medium and high) of seven different combinations of therapeutic agents, providing the most complete assortment of *Antibiotics, Sulfonamides* and other *Agents* for sensitivity testing in

General Infections
GU and GI Infections
TB Therapy

Bacto-Unidisks are sterile, individually sealed in plastic, insuring complete protection and sterility until used. *Each therapeutic agent is identified by name as well as by color.* Concentrations are also clearly marked. Bacto-Unidisks are available in packages of 50.

Bacto-Sensitivity Disks for other therapeutic agents may be used in the center of the Bacto-Unidisk to give additional information.

AVAILABLE ON REQUEST

Bulletin No. 152, describing Bacto-Unidisks
Bulletin No. 146, Microbial Sensitivity Testing
Report Sheets for recording Microbial Sensitivities

DIFCO LABORATORIES
DETROIT 1, MICHIGAN

26A.051
A573

XUM

American Journal of Veterinary Research

Volume XX **January, 1959** **Number 74**

Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

Experimental Infection of Pigs with *Ascaris Suum* 7
B. Schwartz

Use of Scintillation Detector for Determining I^{131} Accumulation in the Thyroid Glands of Swine 14
L. J. Seigneur, L. D. Test, and L. K. Bustad

Anatomic and Functional Studies of the Coronary Circulation in the Dog and Pig 18
G. C. Christensen and F. L. Campeti

Isolation and Characterization of Cytopathogenic Enteroviruses from Cattle with Respiratory Disease 27
T. Moll and A. D. Davis

Feline Infectious Anemia. II. Experimental Cases 33
J. C. Flint, M. H. Roepke, and R. Jensen

Observations on Pyometra in Dogs, with Reference to the Hypothalamic-Hypophysial Neurosecretory System 41
S. Talanti

The Use of Glycine-1-C^{14} in the Measurement of Serum Protein Turnover Rates in Dairy Cows 44
C. E. Cornelius, A. L. Black, and M. Kleiber

A Comparative Study of Lipid Accumulation in the Adrenal Glands of Mature Nonpregnant Dairy Heifers, Nonpregnant Lactating Dairy Cows, and Pregnant Lactating Dairy Cows 53
J. T. Bell and A. F. Weber

(Continued on page 4)

Editors

W. A. Aitken and D. A. Price

Council on Research

Carl A. Brandly, Urbana, Ill.
Charles H. Cunningham, East Lansing, Mich.
Lloyd C. Ferguson, East Lansing, Mich.
Robert Getty, Ames, Iowa
William A. Hagan, Ithaca, N. Y.
Rue Jensen, Fort Collins, Colo.
T. Carl Jones, Boston, Mass.
Hadleigh Marsh, Bozeman, Mont.
Richard D. Turk, College Station, Texas
John D. Wheat, Davis, Calif.

Committee on Journal of the Council on Research

Carl A. Brandly, Lloyd C. Ferguson, William A. Hagan

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953.

Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors.

Subscription price: $10.00 per year in the United States; Canada and Pan American countries, $10.50; foreign countries, $11.00. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $5.00 per year added to the annual dues of $25.00. Single copies $2.50. Address all correspondence to American Veterinary Medical Association.

XUM

Research Journal to Be Published Bimonthly

Starting with this issue, the *American Journal of Veterinary Research* will be published bimonthly, making it possible to somewhat reduce the backlog and also the time lag between receipt and publication of manuscripts. It will, for a while at least, reduce the size of each issue.

In addition, the cover has been redesigned and a new feature, "Scientific Notes," has been added (pp. 223–224).

Additional reviewers have been approved by the Committee on Journal of the Council on Research to aid in the increasing task of reviewing manuscripts. The list of reviewers for the year will appear in the January issue each year.

Reviewers of Research Manuscripts

JOSEPH O. ALBERTS, Urbana, Ill.
JAMES ARCHIBALD, Guelph, Ont.
WILFORD S. BAILEY, Auburn, Ala.
CHARLES N. BARRON, Washington, D.C. (A.F.I.P.)
EDWARD A. BENBROOK, Ames, Iowa
WILLIAM E. BROCK, Stillwater, Okla.
M. LOIS CALHOUN, East Lansing, Mich.
CLARENCE R. COLE, Columbus, Ohio
DAVID W. DETWEILER, Philadelphia, Pa.
ROBERT W. DOUGHERTY, Ithaca, N.Y.
HOWARD W. DUNNE, University Park, Pa.
JEAN C. FLINT, Fort Collins, Colo.
JOHN R. GORHAM, Pullman, Wash.
DONALD P. GUSTAFSON, Lafayette, Ind.
JAMES A. HENDERSON, Guelph, Canada
L. MEYER JONES, Ames, Iowa
WILLIAM KAPLAN, Chamblee, Ga.
ALFRED G. KARLSON, Rochester, Minn.
RALPH L. KITCHELL, St. Paul, Minn.
ROGER P. LINK, Urbana, Ill.
ROBERT R. MARSHAK, Philadelphia, Pa.
CARL OLSON, Madison, Wis.
R. ALLEN PACKER, Ames, Iowa
WILLIAM R. PRITCHARD, Gainesville, Fla.
STEPHEN J. ROBERTS, Ithaca, N.Y.
ARNOLD S. ROSENWALD, Davis, Calif.
JOHN H. RUST, Washington, D.C. (A.F.I.P)
JESSE SAMPSON, Urbana, Ill.
ALVIN F. SELLERS, St. Paul, Minn.
HERBERT G. STOENNER, Hamilton, Mont.
MELVIN J. SWENSON, Ames, Iowa
WALTER G. VENZKE, Columbus, Ohio
CHARLES K. WHITEHAIR, East Lansing, Mich.

(Continued from page 2)

The Comparative Histology of the Skin of Hereford and Aberdeen Angus Cattle..... 61
S. Goldsberry and M. L. Calhoun

Critical Tests with Polymethylenepiperazine (PMP) Against Parasites of the Horse..... 69
J H. Drudge, S. E. Leland, Z. N. Wyant, G. W. Elam, and L. B. Hutzler

The Relationship of Serum Glutamic Oxalacetic Transaminase to Nutritional Muscular Dystrophy in Lambs..... 75
K. F. Swingle, S. Young, and H. C. Dang

Experimental Production of Pneumonia in Lambs..... 78
A. H. Hamdy and W. D. Pounden

Characteristics of a Virus Associated with Lamb Pneumonia..... 84
A. H. Hamdy and V. L. Sanger

Microbial Agents Associated with Pneumonia in Slaughtered Lambs..... 87
A. H. Hamdy, W. D. Pounden, and L. C. Ferguson

Maternally Induced Protection of Young Lambs Against the *Epsilon* Toxin of *Clostridium Perfringens* Using Nonactivated Vaccine..... 91
L. DS. Smith and T. Matsuoka

Septicemic Pasteurellosis in Lambs..... 94
E. L. Biberstein and P. C. Kennedy

Experimental Strongyloidiasis in Sheep and Goats. I. Single Infections..... 102
J. H. Turner

Influence of Temperature on the Growth of Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution..... 111
F. Zuschek, R. P. Hanson, and C. A. Brandly

Titration of Newcastle Disease Virus in Tissue Culture..... 116
D. Durand and A. Eisenstark

Tissue Culture of Avian Visceral Lymphoid Tumors and *in Vitro* Serial Passage of the Virus..... 119
O. S. Davis and D. P. Gustafson

Swine Kidney Cell Cultures—Susceptibility to Viruses and Use in Isolation of Enteric Viruses of Swine..... 127
B. B. Hancock, E. H. Bohl, and J. M. Birkeland

Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus. I. Propagation and Pathogenicity..... 133
R. G. Cunha and E. A. Eichhorn

Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus. II. Immunogenicity..... 138
R. G. Cunha, I. Torturella, and U. M. Serrao

Attempts to Immunize Guinea Pigs with Flury Vaccine and Subsequent Challenge with Fixed Rabies Virus..... 145
C. Huygelen and J. Mortelmans

Characterization of *Vibrio Fetus* Antigens. I. Chemical Properties and Serological Activities of a Soluble Antigen..... 148
M. Ristic and C. A. Brandly

Characterization of *Vibrio Fetus* Antigens. II. Agglutination of Polysaccharide-Sensitized Sheep Erythrocytes by Specific Antiserums..... 154
M. Ristic and C. A. Brandly

Studies on an Immunogenic Agent Against *Brucella Abortus*. I. Titrations of a New Soluble Immunogenic Agent Against Experimental Infections in Guinea Pigs..... 162
W. G. Hoag and R. C. Allen

XUM

Field Evaluation of *Clostridium Botulinum* Type C Toxoids in Mink 166
G. S. Appleton and P. G. White

Laboratory Evaluation of a Trivalent Toxoid for Botulism 170
G. S. Appleton and P. G. White

Studies on *Pasteurella Multocida*. IV. Serological Types from Species Other than Cattle and Swine 173
G. R. Carter

A Serological Study on Some Strains of *Listeria Monocytogenes* Isolated in Michigan 176
J. Donker-Voet

A Pooled Human Serum as a Standard and the Effects of Freezing and Thawing on the Electrophoretic Pattern of Baby Pig Serum 180
R. R. Pensinger, E. F. Reber, E. Ersoy, and H. W. Norton

The Body Fluids of Ruminants and Their Simultaneous Determination 184
E. L. Hix, G. K. L. Underbjerg, and J. S. Hughes

Comparison of Bacteriological and Serological Techniques for Detection of Leptospirosis in Wild Mammals 192
S. McKeever, J. H. Schubert, G. W. Gorman, and R. D. Grimes

Follicular Acariasis of Mice Caused by *Psorergates Simplex* Successfully Treated with Aramite 198
R. J. Flynn

Further Studies on the Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine 201
J. R. Douglas, N. F. Baker, and W. M. Longhurst

Investigations Concerning Bactericidal Substances in Rabbit Uteri 206
H. W. Hawk

Histological Observations of the Genitalia of the Immature, the Castrated, and the Mature Bovine Male 213
D. M. Trotter

Scientific Notes 223

AVMA Research Fellowships Available

The Research Council of the AVMA announces the availability of a number of fellowships for postgraduate training for the academic year, 1959-1960.

The recipient of a fellowship must be a veterinarian and a citizen of the United States or Canada. Veterinary students who expect to graduate at the end of the current school year and who wish to follow a career in research may apply for a fellowship.

The latest date for filing the completed application is Feb. 15, 1959. Approximately one month is required for processing completed applications after receipt by the secretary of the Council. Qualified persons should secure and submit applications as early as possible to insure their file being complete for presentation to the Committee on Fellowships.

The Committee on Fellowships of the Research Council will meet in March to consider applications, and the awards will be announced soon afterward. The stipend will be determined in each case by the needs of the individual, the location of the school in which he proposes to work, and other factors. In general, the stipends range from $100 monthly and upward.

Any qualified person interested in graduate training may obtain application blanks and other information by writing to Secretary, AVMA Research Council, C. H. Cunningham, College of Veterinary Medicine, Michigan State University, East Lansing, Mich.

XUM

FOR
REPRODUCIBLE
RESULTS
IN
INSTITUTIONAL
FEEDING

A foundation product for preparation of menus for feeding laboratory dogs. Available only through graduate veterinarians.

f/d

A "PRESCRIPTION DIET" PRODUCT

PRESCRIPTION DIETS DEPT., HILL PACKING CO., TOPEKA, KANSAS

k/d p/d r/d i/d c/d f/d

American Journal of Veterinary Research

Established 1940

COPYRIGHT 1959 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XX JANUARY, 1959 No. 74

Experimental Infection of Pigs with *Ascaris Suum*

BENJAMIN SCHWARTZ, Ph.D.

Beltsville, Maryland

THIS INVESTIGATION was undertaken primarily to determine the fate of the larvae and the juveniles of *Ascaris suum* which frequently fail to achieve their development, in whole or in part, in their porcine host. Under experimental conditions, pigs appeared to show a resistance to *A. suum*, one of the most common of their internal parasites, that is almost unparalleled in any other natural host-parasite system.

The data recorded here do not account for the fate of the vast majority of the worms which presumably hatched in the intestine of pigs following experimental inoculation with infective *A. suum* eggs. They show, however, that many of these worms were eliminated during their early development, especially while they were undergoing, or had already undergone, the fourth (final) molt and also when they were well along toward development to maturity. The data provide more accurate information than was heretofore available on the growth of *A. suum* in the pig's intestine. This should make it possible to appraise the age of the worms at various stages of development in the intestine of the host, and help interpret the work of other investigators.

REVIEW OF LITERATURE

Since 1916, when Stewart[11] first described the path of migration of newly-hatched *Ascaris lumbricoides* larvae, many workers have corroborated his observations. Stewart's studies on the host-parasite relationship of *A. lumbricoides* and *A. suum* in rats greatly extended the observations made by Davaine half a century earlier. According to Leuckart,[5] Davaine found living Ascaris larvae and others in the process of hatching in the intestines, and living larvae in the feces of rats fed embryonated eggs.

However, in attempts to raise *A. suum* larvae in pigs to maturity, or even to a state approaching maturity, Stewart[11] did not achieve an appreciably greater success, at first, than did Leuckart several decades before him. Leuckart failed to find ascarides in the intestine of a pig fed thousands of embryonated eggs three weeks earlier; he, therefore, concluded that the life cycle of this worm probably involved an intermediate host—a view shared by von Linstow. In experiments carried out by Stewart,[12-15] involving attempts to raise *A. suum* in the intestines of pigs, the seemingly positive results that he obtained in a few cases pointed to a source of infection other than the embryonated eggs which had been administered. Like Leuckart, Stewart considered the possibility that ascarides might have an indirect life cycle, largely because of his failure to raise the larvae beyond 14 days in pigs. In later experiments, he succeeded in obtaining worms up to 7 mm. long from the intestines of pigs fed embryonated eggs 19 days earlier.

Ransom and Foster[7] reported a number of failures and no clear-cut positive results in developing ascarides in the intestine of the porcine host. The most conclusive ecidence they obtained involved hosts other than pigs. A kid, fed *A. suum* eggs 28 and 11 days before necropsy, contained numerous larvae in the lungs, trachea, pharynx, esophagus, rumen, and abomasum, and a significantly larger number of worms in the intestine. In the intestine thousands of worms were present which averaged nearly 8 mm. in length and had developed, presumably, from the eggs of the first feeding, 28 days prior to necropsy.

In another conclusive experiment, with a lamb fed numerous *A. suum* eggs 103 days before necropsy, they recovered 50 partially grown ascarides; the males measured up to 100 mm., the females up to 130 mm. These two experiments

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

Presented before the Parasitology Section of the XV International Congress of Zoology, London, England, July 18, 1958.

demonstrated a direct developmental cycle, even though two unusual hosts were involved.

Martin [9] carried out several series of extensive experiments that involved feeding pigs either *A. suum* eggs or lungs of rabbits and calves which were experimentally infected with Ascaris larvae. Although Martin either failed to find worms at necropsy or recovered only 2 or 3 worms in 6 of 7 pigs fed large numbers of *A. suum* eggs, he discovered 73 worms in the intestine of 1 pig. The size of these worms suggested that they had developed from the inoculum. In another series of tests, using 5 pigs fed rabbit and pig lungs infected with *A. suum* larvae, Martin recovered 52 and 100 worms from 2 pigs and 7, 10, and 17 worms from the remaining 3 pigs. These worms also were of a size that suggested that they came from the inoculum.

Roberts [8] reported the recovery of numerous ascarides from the intestines of 2 pigs killed 14 and 21 days after infection with eggs and only 13 worms from a pig killed 29 days after infection with eggs. In 6 other pigs, killed 34 to 76 days after infection, no worms were recovered from the intestines of 5 pigs, and only 3 worms were recovered from the pig killed 62 days after infection.

De Boer [1] reported negative results in attempts to infect 11 pigs farrowed by a sow that was free of Ascaris. In another trial, the 7 pigs used were farrowed by a sow which passed Ascaris eggs in her feces after anthelmintic treatment and became negative after retreatment. He reported positive results after the administration of eight doses of 5,000 eggs to each of 4 pigs, seven doses of 5,000 eggs to 1 pig, and nearly one half million eggs to each of the other 2 pigs. These doses were given over a period of two to three months. In a third trial, 7 pigs, farrowed by a sow which at no time passed Ascaris eggs, were maintained on a diet deficient in vitamin A to test the hypothesis advanced by a Japanese investigator that pigs on such a diet were susceptible to infection with the human and swine Ascaris. Each of the 7 pigs was given a total of 49,000 *A. suum* eggs in eight divided doses. No ascarides were found when these pigs were necropsied, 45 days after the first dose.

Spindler [10] fed each of 4 pigs daily doses of 12,000 Ascaris eggs for 11 days in succession, then for an additional seven days after a week's rest. About four months later these pigs, each exposed to over 200,000 eggs, harbored 109, 39, 20, and 12 worms, respectively.

Kelley [4] reported failure to infect 6 pigs, 18 days old, after inoculating each pig with 30,000 embryonated eggs of *A. suum*. These pigs had been obtained by hysterectomy, deprived of the sow's colostrum, and maintained on a milk diet. Although the pigs showed respiratory difficulty, none showed Ascaris eggs in their feces, and all were free of Ascaris when necropsied 86 days after inoculation. Seventeen pigs, also obtained by hysterectomy and deprived of colostrum, were placed on a diet of rolled oats when 8, 14, or 35 to 65 days old. They became infected and, when necropsied 22 to 75 days after inoculation with 100,000 embryonated eggs, each harbored an average of 139 worms in the intestines.

MATERIALS AND METHODS

In trials that involved experimental infection with *A. suum*, the pigs used were farrowed by sows which either were initially free of these worms or were freed of them before farrowing. One to two weeks before farrowing, the sows were brought into a sanitary barn. Beginning about 12 to 24 hours after farrowing and daily thereafter until weaning time, the sow and pigs were alternated between two adjacent, communicating pens. These pens were concrete throughout. The unoccupied pen was thoroughly cleaned, first with a stream of cold water, then with hot water, and allowed to dry overnight.

The pigs remained free of Ascaris when removed immediately after weaning to clean, outdoor, individual pens which had concrete floors several feet above the ground. No extraneous infection was discovered in any of the pigs involved in these trials.

One week after the pigs were experimentally infected with *A. suum* eggs or fed lungs from guinea pigs, the feces from each pig were collected and examined daily for eggs. The examination for larvae, beginning about three weeks after inoculation, was made with the aid of screens with meshes of various sizes. The finest mesh retained larvae only a few millimeters long.

EXPERIMENTAL PROCEDURE AND RESULTS

Experiment 1.—On the day of weaning, when 56 days old, pigs 1023, 1024, and 1025 (litter mates) were each given more than 10,000 *A. suum* eggs by stomach tube. Of these eggs, 7,500 were embryonated and approximately 2,800 nonembryonated. The eggs had been cultured for 43 days at room temperature in a 0.5 per cent solution of formalin. Examination of the pigs' feces, beginning 24 hours after infection, showed that a large part, if not all, of the nonembryonated eggs were eliminated by pigs 1024 and 1025 within two days. During this time, no eggs were observed in the feces of pig 1023. Eight to 12 days after feeding the eggs, the pigs eliminated 8, 63, and 2 larvae, respectively. No other worms were found in the feces until 21 to 22 days after experimental infection. Pig 1023 eliminated 872 immature worms 22 to 43 days after infection; 218 was the largest number of worms passed in one day. Pig 1024 eliminated 28 immature worms 23 to 38 days after infection; 13 was the largest number of worms passed in one day. No other worms were discovered in the feces for

the ensuing 30 days. Both pigs began eliminating worms again at 64 days after infection, with pig 1023 passing 28 worms in 12 days and pig 1024 eliminating 38 in 6 days. Again, beginning 104 days after infection, pig 1023 eliminated 4 worms in eight days; pig 1024, beginning 105 days after infection, eliminated 12 worms in three days. The total number of worms passed by pig 1023 was 913; pig 1024 passed 141 worms.

Pig 1025 began passing worms 21 days after infection, one day earlier than the other 2 pigs. The spontaneous elimination of worms continued uninterruptedly for 50 days; 837 was the largest number of worms passed in one day. A total of 1,790 worms passed in this period which corresponds closely to the termination date of the second passage of worms from the other 2 pigs. No more worms were found in the feces of pig 1025.

In general, the length of the worms passed by the 3 pigs gradually increased with their ages. This was particularly true after they had reached an age of 35 days within the host. The average length of 17 worms eliminated 24 days after infection was 9 mm.; after 35 days the average length was 60 mm. and after 43 days it was 84 mm.

Eggs were first discovered in the feces of each of the 3 pigs 49 days after experimental infection. They continued to be eliminated, at first in gradually increasing numbers, until the date of necropsy which, for pigs 1023 and 1024, was 84 days after the eggs were first dropped and which was was one day earlier in pig 1025. At necropsy, all pigs harbored adult worms of both sexes. Those in pig 1025 consisted of 14 females up to 39.0 cm. long and 13 males up to 21.0 cm. long; in pig 1024, 27 females up to 34.0 cm. long and 13 males up to 24.0 cm. long; and in pig 1023, 15 females up to 32.5 cm. long and 16 males up to 23.0 cm. long (table 1).

Experiment 2.—On the day of weaning, when 52 days old, pigs 1065, 1067, and 1069 (from a litter of 8 pigs) were each given, by stomach tube, about 10,260 eggs from a culture prepared 83 days earlier. The culture medium was tap water with no preservative added. Approximately 10,000 of the eggs given to each pig were embryonated. Although the feces of the sow which farrowed these pigs were free of Ascaris eggs, they contained the eggs of Oesophagostomum, estimated at about 18 per gram. No Oesophagostomum eggs were found at any time in the feces of the pigs used in this experiment.

One unembryonated egg and several that were partially or fully embryonated were found in the feces of pig 1069 on postinfection days 3 and 5, but during the first few days no eggs of any kind were found in the feces of the other 2 pigs.

On postinfection day 22, pig 1065 passed 38 immature worms. This pig continued to pass worms during the next 26 days, with the exception of five days, three of which were consecutive. A total of 295 worms were passed; the largest number of worms passed in one day was 50, and the lowest, 1.

Pig 1067 began passing worms 25 days after infection and eliminated 11 worms during a period of 17 days. Pigs 1069 passed a total of 3 worms 30, 32, and 47 days after inoculation. The worms increased in size as they became older. The average length of the worms from pig 1065 on postinfection day 25 was 7 mm.; on day 35, 25 mm.; and on day 45, 56 mm.

Eggs were discovered in the feces of pigs 1065 and 1067 on postinfection day

TABLE 1—Data on Weanling Pigs Infected with Ascaris Suum

	Spontaneous worm passage		Prepatent period			Necropsy findings	
						Length of worms	
Pig No.	Postinfection day	No. passed	(No. of days)	Postinfection day	No. of worms	Females (cm.)	Males (cm.)
1023	22	913	49	133	31	16.0–32.5	11.0–23.0
1024	23	141	49	133	40	17.5–34.0	15.0–24.0
1025	21	1,790	49	132	27	21.0–39.0	12.0–21.0
1065	22	295	50	87	75	20.5–30.0	15.0–20.5
1067	25	11	50	51	109	7.0–17.5	7.5–14.0
1069	30	3	52	87	31	22.5–28.0	15.5–19.5
1064	25	33	47	84	115	19.0–30.0	13.0–22.5
1066	23	448	51	67	204	10.0–23.0	10.5–17.5
1068	....	0	....	74	1	24	.....

50, and in the feces of pig 1069 on postinfection day 52. No eggs had been seen in a salt flotation examination of the feces of pigs 1065 and 1067 on postinfection day 45, or of the feces from pig 1069 on day 48.

Pig 1065, necropsied on postinfection day 87, harbored 75 worms; 37 were females, the longest measuring 30 cm., and 38 were males, the longest measuring slightly over 20 cm. On the same day, pig 1069 had 31 worms; 18 were females, up to nearly 30 cm. long, and 13 were males, up to 19.5 cm. long. Pig 1067 died on postinfection day 51, the day after eggs were found in its feces. Of the 109 worms found at necropsy, the 65 females were up to 17.5 cm. long and the 44 males, up to nearly 14 cm. long.

Experiment 3.—Pigs 1064, 1066, and 1068, litter mates of those used in experiment 2, were infected with Ascaris larvae by feeding each pig one third of a pooled sample of the lungs of 9 guinea pigs which had been given approximately 7,500 embryonated Ascaris eggs 7 days previously. At necropsy, the lungs of the guinea pigs showed extensive hemorrhages and other evidence of infection with Ascaris larvae; 9 larvae were found in a few shreds of lung tissue in press preparation. While these pigs were under observation, no worm eggs, other than those of Ascaris, were found in their feces at any time.

Pigs 1064 and 1066 began eliminating ascarides 25 and 23 days after infection. In approximately 11 days, pig 1064 eliminated 30 worms and pig 1066 eliminated 445 worms. Each of these pigs passed 1 worm on three other occasions, spaced days apart. At necropsy of pig 1064, 84 days after infection, 115 adult worms were found; the longest of the 78 females was 30 cm., and the longest of the 37 males was 22.5 cm. Pig 1066, necropsied 67 days after infection, harbored 204 grown ascarides of which 172 were females measuring up to 23.0 cm. and 32 were males measuring up to 17.5 cm. The worms passed 23 to 25 days after infection were 7 to 9 mm. long, and those passed 45 days after infection were 41 to 64 mm.

Ascaris eggs were found in a salt flotation preparation of the feces of pig 1064 on postinoculation day 47 and in the feces of pig 1066 on day 51 but not on postinoculation days 47 and 49.

Pig 1068, which was kept under observation for 74 days, eliminated no worms or eggs at any time. At necropsy it harbored a single female Ascaris, somewhat over 24 cm. long. Numerous elongated, nonfertilized eggs were found in the uterus of this female.

DISCUSSION

The data show that 6 newly weaned pigs, each given an inoculum of 7,500 to 10,000 infective *A. suum* eggs, developed a patent infection. Two of 3 pigs fed lungs of guinea pigs that were infected a week earlier with the same species also became infected and harbored relatively large numbers of worms; the third pig harbored a single female worm.

In each pig, the size of the worms removed at necropsy was in keeping with their life span in the pigs' intestines. The number of worms recovered was large, with one exception.

There can be little room for doubt, therefore, that the newly weaned pigs used in these experiments were susceptible to worms in the intestines, as well as to larvae in the lungs and livers, especially when the amazing regularity of the first appearance of eggs in the feces, approximately seven weeks after infection, is considered. In the intestine the worms developed up to, and far beyond, egg-laying maturity. Whether the failure of some other investigators to raise *A. suum* to maturity and beyond in the intestines of pigs was due to their attempts to infect suckling pigs, to the inoculation of pigs older than those involved in the present experiments, or to some other cause, can not be stated.

The elimination of worms on a significant scale, other than that which occurred shortly after the return of the larvae from the lungs to the intestine, usually did not become evident until about four weeks after infection. The time when this elimination was first noticed was too uniform to result from chance. It must be concluded that the natural elimination of ascarides from the test pigs is highly significant, even though it can not yet be satisfactorily explained.

The period of 21 to 30 days before the test pigs passed worm corresponds roughly to the beginning of the fourth, or final, molt of *A. suum*. Roberts [8] found what were presumably fourth-stage larvae in the intestine of a pig 14 days after it had

XUM

been infected with the eggs. These larvae, up to 3.2 mm. long, showed morphological development beyond that of the third-stage larvae in the lungs. After 21 days, Roberts found that although some larvae in the intestine of a pig had attained a maximum length of 6.5 mm., their morphological status still remained unchanged from that seen a week earlier. During the eight days that followed the 21-day period, he found worms in the fifth stage, some with a maximum length of 25.5 mm., and others, up to 22.5 mm., still in the process of casting off their final skin. These sizes correspond with those of the large worms eliminated approximately 28 days after infection of the pigs involved in the present experiments.

Less is known of the molting process within the host than of that occurring during the preparasitic development. During the free-living stages of strongylid nematodes the molt is preceded by a lethargus, when a considerable mortality of the larvae may take place. On the assumption that something corresponding to a lethargus also precedes molting within the host, it would appear logical to assume that the beginning of the spontaneous elimination of the worms from the intestine is associated with the molting process, and may even be a consequence of this molting process.

Another intriguing hypothesis is that the elimination of worms which occurred in most of the pigs may have been brought about as a consequence of the development of an immunity produced by the invasion of newly hatched Ascaris larvae of the liver, lymph glands, and other organs.

A recent paper by Taffs [16] reports that, in common with guinea pigs and rabbits, pigs showed antibodies in their serums as a result of infection with Ascaris. These antibodies, demonstrable by the conglutination-complement absorption test, appeared in the circulation of pigs in 10 days and reached a peak approximately 21 days after inoculation. This peak corresponds to the beginning of the spontaneous elimination of the juvenile ascarides recorded here. Although larvae were eliminated by only 2 of 3 pigs that were fed infected guinea pig lungs, 1 pig began passing larvae 26 days after infection, and the other pig began to pass them three days later. In both instances there was a delay, as compared to the beginning of the passage of worms from 5 of 6 pigs infected with the embryonated eggs.

Theory of the Ascaris Life Cycle

The early migratory course of Ascaris larvae is difficult to explain on any basis other than that it presents a panoramic view of the evolutionary history of these worms. Certainly, the roundabout journey of the larvae from the intestine to the lungs and back again to the intestine does not appear to be an adaptation that has survival value for the species under consideration. On the contrary, this migration results in the dispersal of the parasites through the body, inside as well as outside of the hepatic-pulmonary and the lymphatic-pulmonary routes, and into organs and tissues from which they probably cannot extricate themselves.

In order to account for what he considered the "superfluous" migratory route of these larvae, Fülleborn [2] suggested that Ascaris might have evolved from ancestral forms that penetrated the skin. Later he advanced a more logical hypothesis, suggesting that this migration of the larvae probably points to the derivation of these worms from ancestral forms that migrated in an intermediate host.[3] If this hypothesis is accepted, a gradual substitution of the definitive host for the intermediate host can easily be visualized. Projecting this line of reasoning a bit further, the entire development can be conceived of as having become compressed into a single host such as the pig or, in the case of *A. lumbricoides*, man, in which the parasite still behaves more like a heteroxenous than a strictly monoxenous nematode.

The same investigators who reported failures in their attempts to develop Ascaris in the intestines of pigs had no difficulty in raising the parasites in pigs to the stage that they attain in the lungs and, to some extent beyond that, in the intestines. There is practically no known mammalian host specificity in the early development of Ascaris. *Ascaris lumbricoides*, as well as *A. suum*, has repeatedly been shown to produce signs of pulmonary changes in pigs and other mammals. Host specificity becomes evident only when the worms return to the intestine. In small mammals such as mice, rats, guinea pigs, and rabbits, *A. suum* passes out of the body shortly after returning to the intestine. In goats, and more so in sheep and cattle, *A. suum*

XUM

can grow in the intestine and may attain about half of its normal size. Half-grown *A. suum*, the females containing eggs that could be cultured to the embryonated stage, were found in a calf.[9]

The behavior of *A. suum* in pigs often parallels its behavior in a strange host. This presents an anomaly that is difficult to explain, except by postulating the development of a marked resistance to these worms by the host. This resistance could occur particularly during the period when the larvae wander through the body. The fact that pigs can be infected by being fed the lung stage of the worms lends support to the idea that the two phases of the parasite's life cycle are more or less distinct, and that the stage attained in the lungs marks the end of the first developmental phase and the beginning of the second one. The ability of the worms to reach a stage of development in the lungs of the host that is preparatory to the second phase is no guarantee that their subsequent development in the intestine will take place. Much still remains to be determined regarding the factors that render some pigs susceptible and others resistant to the development of *A. suum* in their intestines to a stage of egg-producing maturity.

SUMMARY

Patent infections with *Ascaris suum* were produced experimentally in 8 of 9 pigs. Embryonated eggs were fed to 6 of the pigs; the other 3 pigs were fed lungs of guinea pigs similarly infected a week earlier.

The 6 pigs, each fed 7,500 to 10,000 embryonated eggs, harbored 27 to 109 worms of both sexes at necropsy, 51 to 133 days later.

The sizes of the worms recovered at necropsy varied with their ages; the smallest were found 51 days after infection, the largest were found nearly four and one half months after infection. Worms obtained approximately three months after infection were of intermediate sizes.

Two of 3 pigs fed parasitized guinea pig lungs harbored, respectively, 204 and 115 worms of both sexes at necropsy; the third pig harbored a single female worm. The sizes of the worms recovered at different periods varied with their age.

In 2 of the pigs fed parasitized guinea pig lungs, eggs were first discovered in the feces 47 and 51 days after infection; no eggs were eliminated at any time by the pig containing a single female worm. In pigs fed embryonated eggs, the prepatent period varied between 49 and 52 days.

Approximately four weeks after infection, larvae were found in the feces; their number tended to increase gradually over a prolonged period. Five of the pigs eliminated 141 to 1,790 worms; in the remaining 4 pigs the number of worms was much smaller. The naturally eliminated worms showed a gradual increase in size that generally varied with the time they had remained in the intestine.

The natural elimination of the worms appeared to be associated with the final molt. It may also be associated with a resistance developed by the host that was stimulated in part by the penetration of the larvae into various tissues during their early migration.

The early migration of Ascaris larvae does not appear to be an adaptation that has a survival value for the species; on the contrary, it leads worms into situations from which they probably can not extricate themselves.

The migration of the newly hatched larvae appears to be of phylogenetic significance and probably points to the evolution of these worms from ancestral forms that had an indirect life cycle. On the basis of this assumption it may be concluded that the life cycle of *A. suum* and of related species apparently has become compressed into one host, although it still shows some evidence of an indirect development, with one phase culminating in the lungs and the other in the intestine.

References

[1] de Boer, E.: Experimentelle Untersuchungen über *Ascaris Lumbricoides* des Menschen und des Schweines. Ztschr. f. Infektionskr., *48*, (1935): 248–269.

[2] Fülleborn, F.: Über den Inkectionsweg bei ascaris. Klin. Wchnschr., *1*, (1922): 984–988.

[3] Fülleborn, F.: Über das Verhalten der Larven von *Strongyloides Stercoralis*, Hakenwürmern und *Ascaris Lumbricoides* im Körper des Wirtes und ein Versuch, es biologisch zu deuten. Beihefte Arch. Schiffs- u. -Tropen-Hyg., *31*, (1927): 151–202.

[4] Kelley, G. W., Olsen, L. S., and Hoerlein, A. B.: The Influence of Diet on the Development of *Ascaris Suum* in the Small Intestines of Pigs. Am. J. Vet. Res., *19*, (April, 1958): 401–404.

[5] Leuckart, Rudolph: Die menschlichen Parasiten und die von ihnen herrührenden Krankheiten. Leipzig, 1876.

[6] Martin, H. M.: Studies on the *Ascaris Lumbricoides*. Nebraska Agric. Exper. Sta. Bull. 37, 1926.

[7] Ransom, B. H., and Foster, W. D.: Observations on the Life History of *Ascaris Lumbricoides*. U.S.D.A. Bull. 817. 1920.

[8] Roberts, F. H. S.: The Large Roundworm of Pigs, *Ascaris Lumbricoides* L., 1758, Its Life History in

Queensland, Economic Importance and Control. Yeerongpilly Anim. Health Sta. Bull. 1, 1934.

[9] Schwartz, Benjamin: Occurrence of Ascaris in Cattle in the United States. North Am. Vet., *6*, (1925): 24–30.

[10] Spindler, L. A.: The Effect of Experimental Infections with Ascarids on the Growth of Pigs. Proc. Helminthol. Soc. Washington, *14*, (1947): 58–63.

[11] Stewart, F. H.: On the Life History of *Ascaris Lumbricoides*. Preliminary Note. Brit. M. J., *2*, (1916): 5–7.

[12] Stewart, F. H.: On the Life History of *Ascaris Lumbricoides*. Brit. M. J., *2*, (1916): 753–754.

[13] Stewart, F. H.: On the Life History of *Ascaris Lumbricoides* L. Parasitology, *10*, (1918): 197–205.

[14] Stewart, F. H.: On the Life History of *Ascaris Lumbricoides* L. Parasitology, *11*, (1919): 385–387.

[15] Stewart, F. H.: On the Life History of *Ascaris Lumbricoides* L. Part 5. Parasitology, *13*, (1921): 37–47.

[16] Taffs, L. G.: Immunological Reactions of the Host to *Ascaris Lumbricoides*. Vet. Rec., *69*, (1957): 804.

Use of Scintillation Detector for Determining I^{131} Accumulation in the Thyroid Glands of Swine

L. J. SEIGNEUR, D.V.M.; L. D. TEST, M.S.; L. K. BUSTAD, D.V.M.

Richland, Washington

RADIOIODINE (I^{131}) is an important product of atomic fission and is present in the gaseous wastes of chemical processes in the manufacture of plutonium. Contamination of vegetation with I^{131} represents a potential hazard requiring definition.

Long-term biological effects of I^{131} were investigated, using the sheep [1] as a representative grazing animal, to define the maximum level of I^{131} given daily that will not cause thyroid damage. A similar study was initiated with pigs to faciliate better extrapolation to man.

This paper reports initial observations on thyroidal I^{131} accumulation, using Palouse swine* maintained on high and low planes of nutrition. A direct relationship between nutritional level and I^{131} accumulation is shown.

The intractable nature of the pig, variation in depth of thyroid glands, and wide variety of animal size required development of a sensitive thyroid monitor [2] that would perform accurately without animal contact. Instrumentation and associated restraint equipment are described briefly for the benefit of clinicians interested in conducting comparative thyroid metabolism studies.

MATERIALS AND METHODS

All swine were group-fed a prepared, marginally goitrogenic ration (low in iodine) that was periodically analyzed for stable iodine content. Those on a low-level ration were fed 70 per cent, by weight, of the daily allowance for swine on full feed and individual daily supplements of stable iodine to equalize their iodine intake with that of the high-level group. The two nutritional levels have been maintained for several generations.

Gilts averaging 17 months of age, 4 on a high- and 4 on a low-plane ration were used initially in two consecutive I^{131} tracer studies, since their use in the long-term study would preclude further observations of this nature. Each gilt was fed 45 μc. of I^{131} and subjected to thyroid gland monitoring at six-hour intervals for 48 hours and daily thereafter, until less than 1 μc. remained in the thyroid gland. The same procedures were followed in the second tracer study.

Animal age, plane of nutrition, and level of radioiodine fed daily in the long-term study are shown (table 1). The thyroid glands were monitored daily until approximate thyroidal I^{131} equilibrium was indicated, twice weekly for 30 days, and once each week thereafter.

Thyroid monitoring is accomplished with the scintillation detector [2] and restraint equipment shown (fig. 1). Two probes are arranged, 18 inches apart and diametrically opposite, on a rigid yoke mounted on tracks at the top of the forward one third of a pig chute. Each probe contains a sodium iodide (Tl) crystal (1¾ inches in diameter by ½ inch thick) and photomultiplier tube. Counts measured by a counting rate meter and preserved on a strip chart recorder are obtained from the phosphor pulses which have been amplified and differentially discriminated so that only the pulses corresponding to the principal photopeak of I^{131}

TABLE 1—Experimental Design of Chronic I^{131} Study

No. in group	Average age (mo.)	Nutritional plane	I^{131} fed daily (μc.)
4	17	High	45.0
4	17	Low	45.0
4	10	High	5.0
4	10	Low	5.0
4	10	Low	0.5

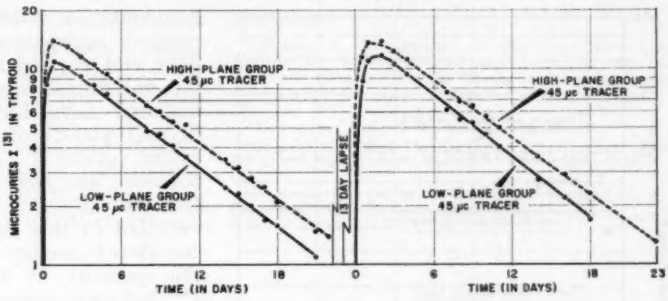
From the Biology Operation, Hanford Laboratories, General Electric Co., Richland, Wash. Dr. Seigneur is Captain, U.S.A.F., V.C., and is now with the Department of Radiation Biology, University of Rochester, N.Y. Mr. Test, formerly of Instrument Research and Development, Hanford Atomic Products Operation, is now with the Atomic Power Equipment Department, General Electric Co., San Jose, Calif.

This paper is based on work performed under contract No. W-31-109-Eng-52, for the Atomic Energy Commission.

The authors acknowledge the consultation services of Drs. M. E. Ensminger, chairman, and S. H. Fowler, Department of Animal Husbandry, State College of Washington, Pullman, and the technical assistance of V. G. Hostman, M. E. Kerr, L. A. George, Jr., W. J. Clarke, and the Experimental Animal Farm attendants.

* The pigs were obtained from the project, "A Fundamental Study of the Relationship of Nutrition to Improvement of Animals for Meat Production Through Breeding," conducted by the Department of Animal Husbandry, State College of Washington, Pullman.

Feed components (in %) were as follows: ground barley, 27.5; ground wheat, 24.0; dehydrated alfalfa, 15.0; soybean oil meal, 14.0; meat scraps, 12.5; millrun, 0.7; dried skim milk, 4.0; corn distiller's dried solubles, 0.6; steamed bone meal, 0.6; ground oyster shell, 0.6; salt (noniodized), 0.52; and mineral mixture, 0.08. Stable iodine analyses of consecutive lots ranged from 0.11 to 0.35 μg. of iodine per gram.



Graph 1—Thyroidal I^{131} uptake and decay in swine on high- or low-level rations.

(0.36 m.e.v.) are accepted. Differential discrimination eliminates the need for heavy shielding which would otherwise be necessary to reduce air background. The instrument has a 1,400 count per minute per microcurie sensitivity and a normal air background of 400 counts per minute.

The pig is trained to enter the chute and eat from an adjustable feed box mounted on the front swing-type door. A panel installed in vertical tracks on the rear adjustment assembly is raised manually for entrance, swings forward as the pig walks under, following the back contour, and drops behind the pig to prevent backward movement. The rear assembly pivots forward from floor level and adjusts to varying body length by seating the ratchet arms at the top of the chute.

The probes are adjusted to thyroid height and the counts obtained while the pig is feeding from the elevated feed box, since this position raises the thyroid gland in front of the point of the shoulder. The shoulder would represent a substantial shielding effect if the thyroid gland were monitored while the pig fed from floor level. A single vertical adjustment of probes usually suffices for all pigs of a given age range. Slight forward and backward movement is compensated for by a similar movement of the probes on the horizontal tracks.

Body background (counts originating from extrathyroidal accumulations of I^{131} in the blood, intestines, and viscera) is determined by enveloping the thyroid gland region with a two-section, detachable, 0.5-inch-thick lead collar (fig. 1) capable of shielding 99 per cent of detectable *gamma* emissions. The open count, obtained by removing the lead collar, less body background,

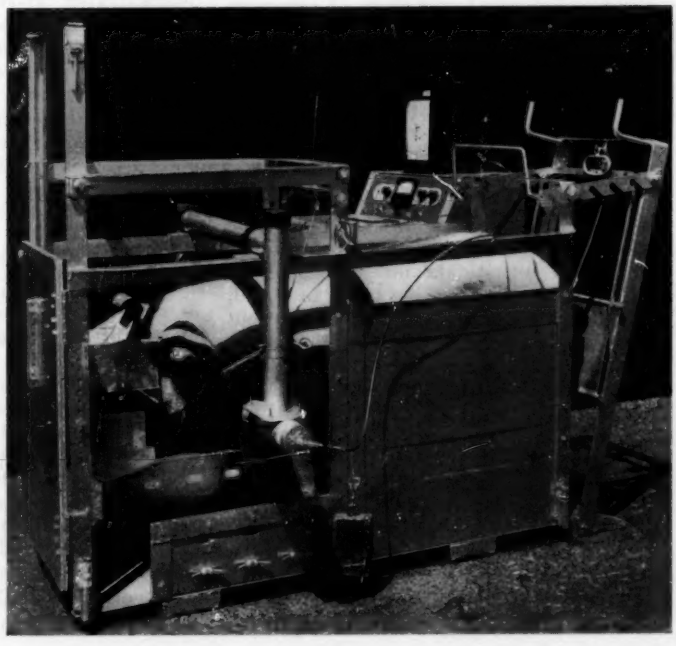
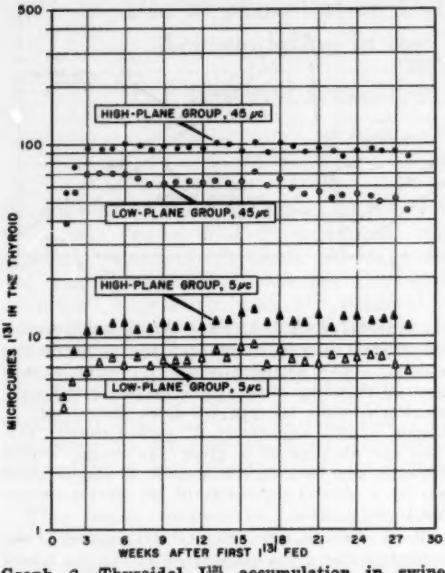


Fig. 1—The two-probe thyroid monitor in use (notice the shields in place).



Graph 2—Thyroidal I^{131} accumulation in swine on high- or low-level rations (I^{131} fed daily).

establishes net counts attributed to thyroidal I^{131}. Apparent microcuries (quantity of I^{131} present, disregarding neck tissue shielding effect) are found by dividing net counts by counts per minute per microcurie from a known source. A conversion factor based on body weight is applied to this quotient, giving actual microcuries in the thyroid gland. Conversion factors are related to depth of thyroid gland beneath the skin surface, measured along the axis connecting the two probes, and were found through slaughter of pigs of different weights with known quantities of thyroidal I^{131}.[2]

Results and Discussion

Tracer I^{131} Uptake and Turnover.—Tracer study comparison of the animals on the two nutritional levels showed no significant differences in time to reach maximum I^{131} accumulation in the thyroid glands or in effective thyroidal I^{131} half-life. Maximum I^{131} in the thyroid glands was evidenced between 20 and 30 hours after feeding, and the effective half-life averaged six days. Maximum absorption coefficients (I^{131} in the thyroid/total I^{131} fed) averaged 0.32 in the swine on the high-level ration, and 0.26 in those on the low-level ration, and the comparative accumulation remained greater in the group on the high-level ration through decay (graph 1). Statistical analysis was not made.

Chronic I^{131} Uptake and Turnover.—Accumulation of I^{131} in the thyroid glands reached approximate equilibrium in 21 days and appeared to be independent of the nutritional level. Appreciably higher amounts have been maintained in the thyroid glands of swine on a high-level ration (graph 2), although the data have not been analyzed statistically. The swine fed the 0.5-μc., low-level ration are not included in this figure, since the level is considered nondamaging and the ratio of the quantity of I^{131} in the thyroid to I^{131} fed daily varies little from that of the swine fed the 5-μc., low-level ration.

Average weights of thyroid glands of the animals on different nutritional levels are not known at this time. Necropsy may reveal larger thyroid glands in animals on the high-level ration, and thus indicate one reason for their greater thyroidal I^{131} accumulations.

The instrument (fig. 1) has been in constant use for more than 18 months and there have been few electronic failures, even though it was exposed to moderately rough treatment and extremes in temperature (20-100 F). Measurements of I^{131} in the isolated thyroid gland have shown excellent agreement with radiochemical analysis, and *in vivo* monitoring has demonstrated good reproducibility when measuring less than 1 μc. of thyroidal I^{131}. The pigs have adapted to a weekly count with no adverse consequences and no supplementary training. The identical instrumentation and restraint equipment with only a neck shield modification have been used successfully in sheep thyroid gland monitoring and may find application with other animals.

Summary

Application of a two-probe scintillation detector and associated restraint equipment for quantitative *in vivo* measurement of I^{131} in the thyroid glands of swine is described in a combined long-term study to determine the effects of nutrition on thyroidal I^{131} metabolism and to define the maximum amount of I^{131} administered daily that will not cause thyroid damage. Initial observations on nutritional effects revealed significantly higher thyroid gland accumulations in the full-fed pigs, as compared with accumulations in pigs fed 70 per cent of the full ration, in both tracer and chronic feedings of the isotope.

Both levels of nutrition attained maximum thyroidal I^{131} accumulations between 20 and 30 hours after single feedings of the isotope, and the effective thyroidal I^{131} half-life averaged six days.

Chronic I^{131} feeding showed approximate equilibrium accumulations in the thyroid glands in 21 days and the time appeared to be independent of the nutritional level.

References

[1] Bustad, L. K., George, L. A., Jr., Marks, S., Warner, D. E., Barnes, C. M., Herde, K. E., and Kornberg, H. A.: Biological Effects of I^{131} Continuously Administered to Sheep. Radiation Res., *6*, (1957): 380–413.

[2] Seigneur, L. J., Test, L. D., and Bustad, L. K.: Scintillation Detector for Thyroidal I^{131} in Pigs. Biol. Res., Ann. Rep., 1955, Document HW–41500, (1956): 166.

[3] Test, L. D., Seigneur, L. J., and Bustad, L. K.: Pig Thyroid Monitor. Hanford Atomic Products Operation. Document HW–48712, 1957.

XUM

Anatomic and Functional Studies of the Coronary Circulation in the Dog and Pig

GEORGE C. CHRISTENSEN, D.V.M., Ph.D., and FRANK L. CAMPETI, M.D.

Ames, Iowa, and Rochester, New York

PIONEERING surgical techniques of recent years have established the necessity for precise comparative anatomic knowledge of the heart. A study of the coronary vessels in domestic animals is requisite to the understanding of cardiac defects in these species; its implications are important in man. A controlled investigation of experimentally produced changes in the hearts of litter mates (dogs or pigs), living under similar conditions and given specific diets, is being conducted by the senior author.

This report, concerning a segment of the comprehensive investigation, is limited to the morphological and functional patterns of coronary circulation in the dog and pig. The cardiovascular architecture of these species differs with regard to the comparative aspects of atrial arteries, the distribution of terminal ventricular arteries, and venous drainage. Cineangiocardiographic studies of living animals are included which show the functional pattern of coronary circulation rather than detailed angioarchitecture.

MATERIALS AND METHODS

Vinyl acetate and chloroprene latex (Neoprene*) casts, clearings, and serial sections of 400 hearts were used to establish the detailed pattern of intrinsic cardiac vasculature, following procedures described by the senior author.[3, 4] Litter mate pigs and purebred Beagles were used.

Cineangiocardiographs of dogs and pigs were made at the University of Rochester, School of Medicine and Dentistry, Rochester, N.Y. With the animal under general anesthesia, the right carotid artery was cannulated with a balanced polyethylene tube. The free end of the cannula had been previously closed and the distal 2 cm. was perforated with six holes. It was perfectly balanced to assure even distribution of radiopaque material to both coronary arteries. A steady drip of saline solution into the tube prevented the backflow of blood. By injecting 1 ml. of 70 per cent sodium acetrizoate, with simultaneous fluoroscopic examination, the distal end of the cannula was put into the ascending aorta at the level of the coronary arteries (fig. 1).

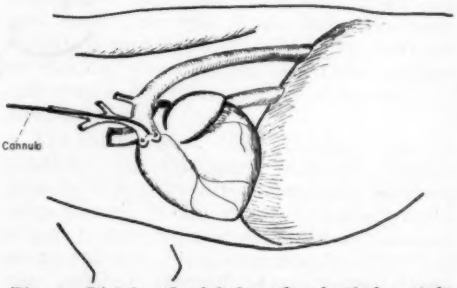


Fig. 1—Distal end of balanced polyethylene tube positioned at level of aortic valves. This permits even distribution of contrast material to both coronary arteries.

Prior to x-ray exposure, the tubing containing saline solution was detached from the cannula, and the free end of the cannula was attached to a record syringe containing 15 to 25 ml. of 70 per cent sodium acetrizoate. During x-ray exposure, the sodium acetrizoate was injected for four seconds, under a pressure of 20 to 65 lb. per square inch. The pressure and injection time was varied according to the size of the animal, the comparative resistance of arteries and polyethylene tubing, and the total amount of radiopaque solution. One milliliter of 70 per cent sodium acetrizoate was injected for each kilogram of body weight.

The cineangiocardiography unit, designed by Gramiak *et al.*,[6] is a 35-mm. camera fitted with a high quality 0.71 f lens. The film-transport mechanism is adjustable for film speeds of 3¾, 7½, 15, or 30 frames per second, and is powered by a synchronous motor that drives the shutters and regulates exposure time. Counter-rotating shutters

From the Department of Veterinary Anatomy, Division of Veterinary Medicine, Iowa State College, Ames (Christensen), and the Department of Radiology, School of Medicine and Dentistry, University of Rochester, Rochester, N.Y. (Campeti). Dr. Christensen is now professor and head, Department of Veterinary Anatomy, School of Veterinary Science and Medicine, Purdue University, Lafayette, Ind.

Presented before the Section on Research, Ninety-Fifth Annual Meeting, American Veterinary Medical Association, Philadelphia Pa., Aug. 18–21, 1958.

This investigation was supported by research grant H-2597 from the National Heart Institute, Public Health Service.

The authors acknowledge the counsel of Drs. M. Emmerson and R. Getty, Division of Veterinary Medicine, Iowa State College, Ames.

* Neoprene is produced by Cementex Co., New York, N.Y.

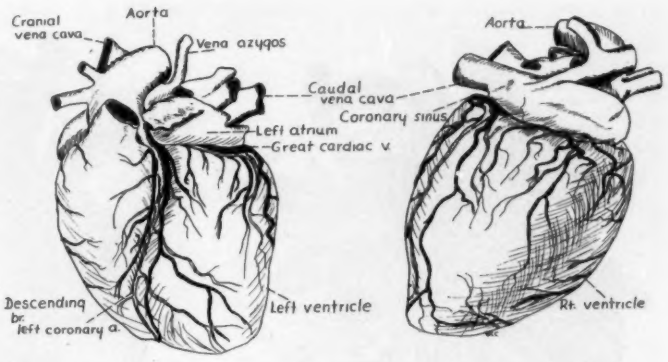


Fig. 2—Superficial views of the canine heart, illustrates the surface pattern of coronary arteries and veins. Left and right views of the heart are represented.

in front of the lens provide a positive cutoff of light from the E-2 fluorescent screen and prevent fogging of the film due to screen persistence during transport. The exposure-timing control is governed by a 180-degree commutator segment driven by the camera gear train.

Most of the films were made at 30 frames per second. For viewing and study, 16-mm. reduction prints were made. Prior to filming, 20 to 25 mg. of acetylcholine and 1 to 2 mg. of dihydroergotamine 45 were injected in some animals to slow heart action. Exposure time was 11 seconds, using 85 ma. and 100 kv. The latter two figures varied according to the size of the animal. Dogs weighed 15 to 30 lb.; pigs weighed 15 to 60 lb. Later studies were made using the image-amplifier tube, filming at 15 frames per second and using lower milliamperage.

Observations

Vinyl acetate and chloroprene latex casts showed morphological differences in the arrangement of arteries and veins in the heart of the dog and that of the pig. In the dog, there are three major atrial arteries from each of the coronary arteries: right and left ventral, dorsal, and intermediate atrial arteries, in agreement with Halpern.[7,8] The right ventral branch supplies the ventral aspect of the right auricle and atrium. It gives off the accessory ventral right atrial artery, which supplies arterial blood to the right atrium and to the aortic wall at the level of the valves. Its numerous terminal branches supply the interatrial septum, the common bundle, and the atrioventricular node. Many dogs have a separate artery with this same distribution.

The left ventral atrial artery, arising close to the origin of the left coronary artery, supplies the ventral side of the left auricle, the left atrium, and the interatrial septum.

The dorsal left and right atrial arteries, arising from the terminations of the circumflex coronary arteries, help supply blood to the interatrial septum. An accessory ventral left atrial artery, present in

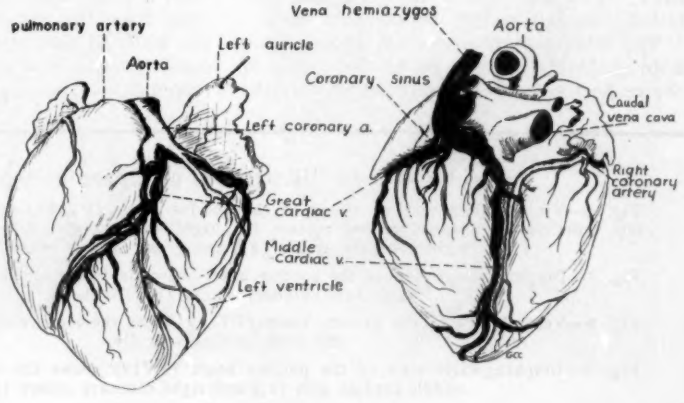


Fig. 3—Superficial views of the porcine heart—left ventral and right diaphragmatic views.

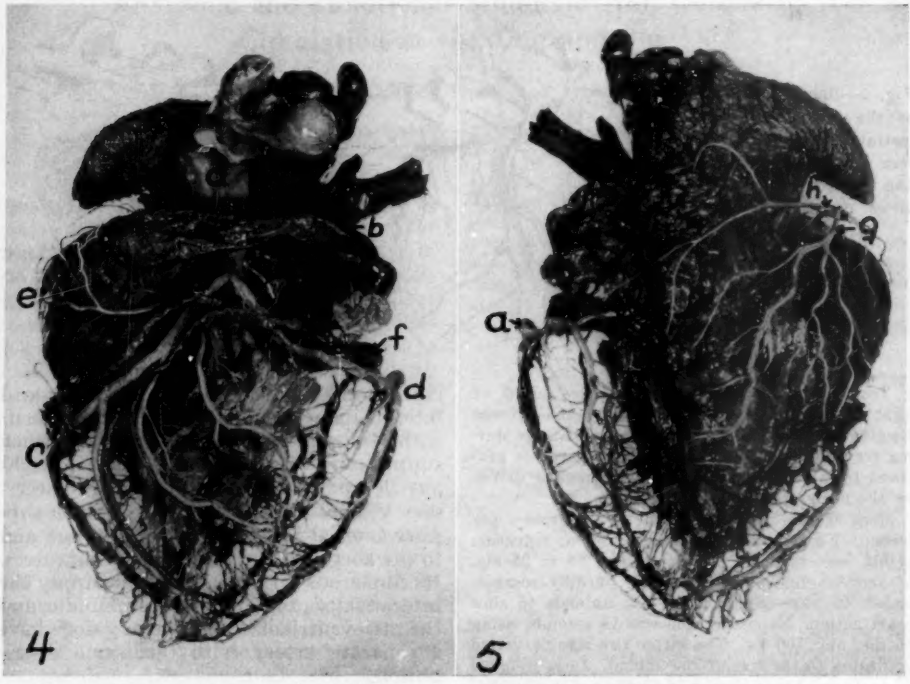


Fig. 4—Vinyl acetate cast of vasculature of the canine heart (DV27), left side, shows the aorta (a), pulmonary artery (b), descending branch of left coronary artery (c), circumflex branch of left coronary artery (d), ventral right atrial artery (e), and great cardiac vein (f).

Fig. 5—Diaphragmatic view of the canine heart shown in figure 4. The left coronary artery (a), right coronary artery (g), and dorsal right atrial artery (h) are shown.

some dogs, supplements or replaces a variable accessory ventral right atrial artery in supplying the interatrial septum. The dorsal left atrial artery supplies the walls of the pulmonary veins and the caudal vena cava; its counterpart on the right side contributes largely to the cranial vena cava.

The intermediate atrial arteries, located approximately midway along the course of the respective coronary arteries, supply the adjacent atrial musculature. Small atrial arteries, too numerous and variable to be named, arise along the entire course of the parent vessels.

The pig has fewer large atrial arteries than the dog, but numerous minute vessels arise from the coronary arteries to supply the walls of the upper heart chambers. It consistently has a large artery which corresponds to the ventral left atrial artery

Legends for Illustrations on Opposite Page

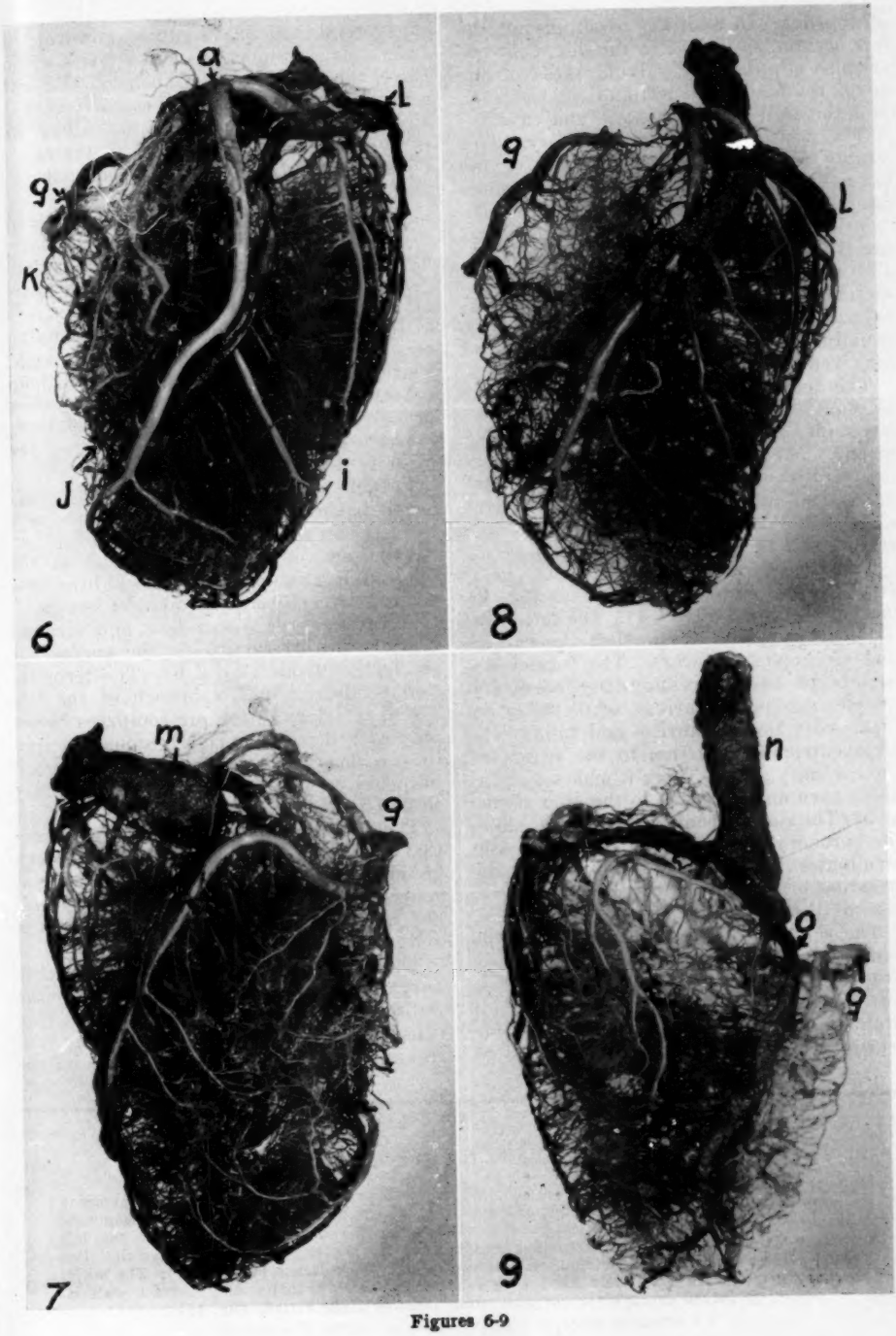
Fig. 6—Vinyl acetate cast of vessels of the porcine heart (PV19), ventral view, shows the left ventricle (i), interventricular septum (j), right ventricle (k), left coronary artery (a), right coronary artery (g), and great cardiac vein (l).

Fig. 7—Diaphragmatic view of the porcine heart shown in figure 6. The coronary sinus (m) and right coronary artery (g) are shown.

Fig. 8—Ventral view of the porcine heart (PV13) shows the right coronary artery (g) and the great cardiac vein (l).

Fig. 9—Diaphragmatic view of the porcine heart (PV13) shows the vena hemiazygos (n), middle cardiac vein (o), and right coronary artery (g).

XUM



Figures 6-9

XUM

of the dog. In addition to supplying the left atrium, as it does in the dog, this vessel also supplies the valvular area of the aorta, the base of the left and right atrium, and the wall of the cranial vena cava.

The ventral right atrial artery helps supply the right atrium, the base of the cranial vena cava, the walls of the pulmonary veins, and the caudal vena cava. Intrinsically, these vessels supply the interatrial septum, the atrioventricular node, and the common bundle, along with numerous small branches from the coronary arteries. The ventral right atrial artery usually arises in common with the accessory ventral right artery.

The dorsal right atrial artery in the pig originates from the distal one third of the circumflex branch of the right coronary artery. The dorsal left artery frequently arises in a common trunk with the ventral left atrial artery, supplying the ventral side of the left atrium, the aortic valve region, and the pulmonary veins.

Ventricular arteries in the dog arise from the two coronary arteries leaving the base of the aorta (fig. 2, 4). The left coronary artery gives off the left descending and circumflex branches. The former descends in the corresponding coronary groove (sulcus coronarius), while the latter dips under the left auricle and follows the atrioventricular junction to the region of the coronary sinus. There it makes a right-angle turn and descends to the apex of the heart. The right coronary artery runs along the coronary sulcus of the right side and terminates in a comparatively small descending branch on the diaphragmatic surface of the heart.

The septal artery arises at or near the junction of the descending and circumflex branches of the left coronary artery. After 1 to 3 cm., it terminates in numerous small branches supplying the interventricular septum. The principal source of blood to the interventricular septum, however, is from a large number of small arteries which leave the descending branch of the left coronary artery and, to a lesser extent, the right, to enter the septum all along its periphery. This also occurs in the heart of the pig and other domestic animals, as well as that of man (fig. 4–9).[8]

The right circumflex coronary artery of the pig, unlike that of the dog, terminates by descending in the longitudinal sulcus of the diaphragmatic surface of the heart (fig. 3, 6, 8). The right circumflex artery of the pig also gives off a large descending branch to the central portion of the right ventricle. As in the dog, numerous small branches arise from the right circumflex branch along its entire route and contribute arterial blood to the musculature of the right ventricle.

Porcine hearts differ greatly from canine hearts in the manner by which vessels leave the left coronary artery to supply the corresponding ventricle. In addition to the descending circumflex and septal branches, a large artery leaves the area of the junction between the descending and circumflex arteries to ramify on the surface of the left ventricle (fig. 3, 6). The termination of the circumflex branch of the left coronary artery in the pig compares favorably with that of the right coronary artery in the dog. It ends in numerous small branches in the region of the coronary sinus (fig. 3).

The heart of the pig also differs from that of the dog with regard to the pattern of venous drainage (fig. 3, 6–9). The atrial veins in both species are relatively small and numerous, empty into the right atrium and, in the pig, into the coronary sinus.

The cardiac veins accompany the coronary arteries and return blood from the walls of the heart. The great cardiac vein runs in the longitudinal sulcus on the ventral surface of the heart, passes around the

Legends for Illustrations on Opposite Page

Fig. 10–15—Stills from a cineangiocardiograph show 70 per cent sodium acetrizoate pumping through the coronary vessels of a 6-month-old, 29-lb. Collie dog (D4A). Total injection time was three seconds. Start of injection shows filling of the descending branch of the left coronary artery (a) and the circumflex branch (b) during left ventricular diastole. One second after beginning the injection, an atrioventricular block occurs (fig. 12–14). The major coronary arteries are completely filled with contrast material. Finally, the contrast material is forced through the coronary sinus into the right atrium (fig. 15).

c = coronary sinus, d = aortic valve, e = aorta, f = cannula

XUM

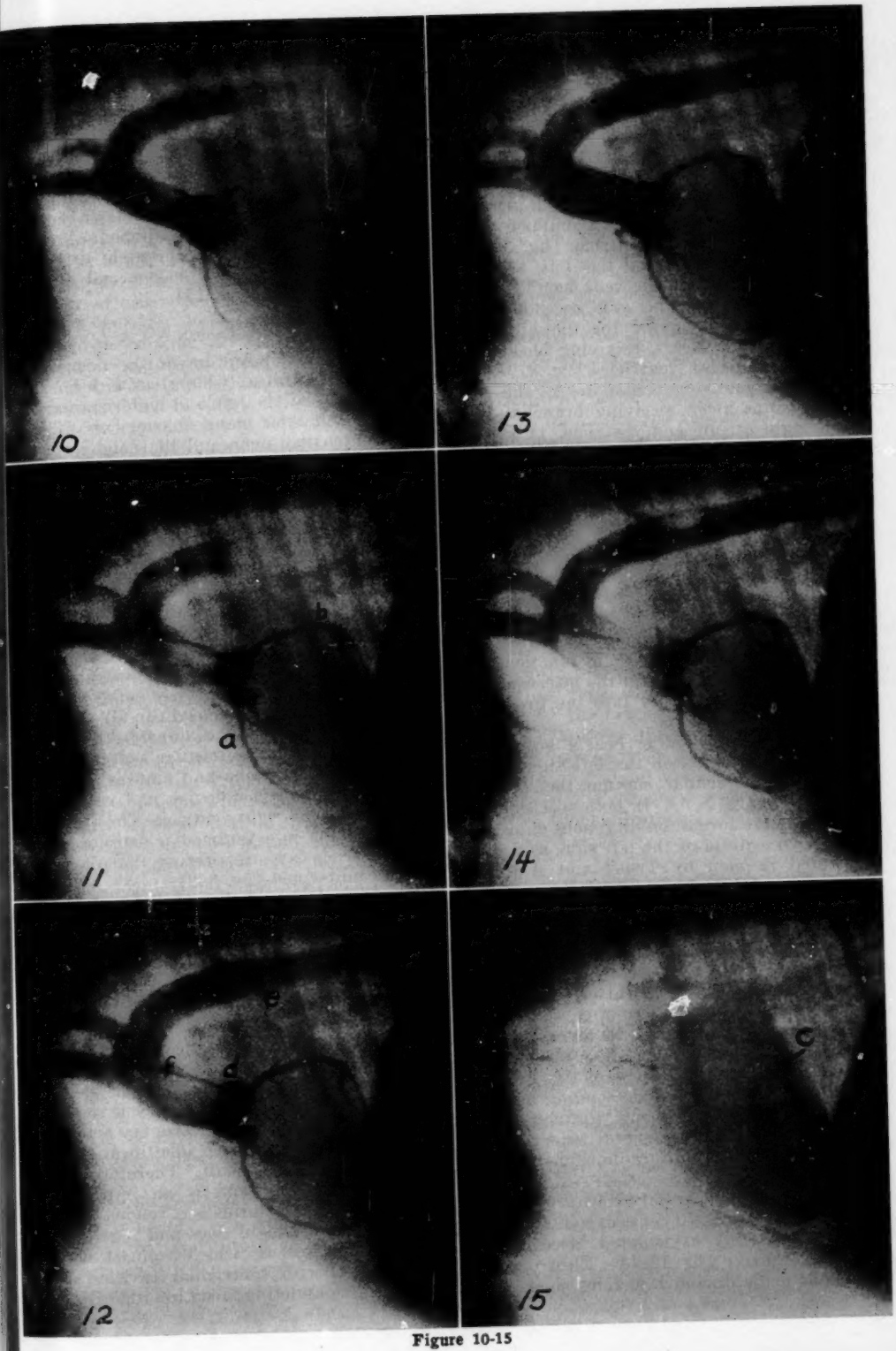


Figure 10-15

left side of the heart in the coronary sulcus, and terminates in the coronary sinus (fig. 2–4, 6, 8). The coronary sinus of the dog is small; in the pig, a large sinus is located at the junction of the vena hemiazygos with the great and middle cardiac veins and with numerous tributaries from the left atrium and ventricle (fig. 3, 7, 9). Numerous veins from the left ventricle drain directly into the vena hemiazygos.

The middle cardiac vein ascends in the longitudinal sulcus of the diaphragmatic surface of the heart, receiving blood from the walls of both ventricles (fig. 3, 9). The small cardiac vein terminates in the coronary sinus after receiving branches from the right atrium and ventricle. Other cardiac veins include the anterior cardiac veins and the posterior vein of the left ventricle. The former arise from the right ventricle and drain into the right atrium; the latter drains the left ventricular wall and opens directly into the coronary sinus.

In porcine hearts, relatively large septal veins have been found emptying into the great cardiac veins. In some pigs, the anterior and great cardiac veins unite and receive septal veins at their junction. Numerous small veins drain the interventricular septum and empty into the great and middle cardiac veins.

Canine and porcine hearts have more arteries and veins on the left side and in the interventricular septum than on the right side (fig. 8, 9). In both species, there is a massive formation of venous sinuses in the myocardium of the left side, as demonstrated in man by Truex and Angulo.[13] Luminal (thebesian) vessels[11] in vinyl-injected hearts demonstrate direct continuity between the venous sinuses of the myocardium and the lumina of the ventricles.

Because of physiological errors in postmortem injection techniques, cineangiocardiography was used to determine the principal routes of blood flow in hearts of living dogs and pigs. These motion pictures indicated that the left descending and circumflex coronary arteries, the right coronary artery, and the septal artery simultaneously filled with radiopaque solution (fig. 10–15).

When the myocardial arteries were completely filled with 70 per cent sodium acetrizoate, replacing oxygenated blood,[4] heart blocks occurred (fig. 12–14). Electrocardiographs made during injections showed the existence of heart blocks; they will be discussed in detail in a later publication. Repeated injections, even after a 30-minute interval which allowed the animal to recuperate and the x-ray tube to cool, resulted in myocardial and renal infarcts in both dogs and pigs. When the radiopaque solution started to flow into the right atrium through the coronary sinus, normal heart function resumed (fig. 15).

DISCUSSION

The interatrial septum of the dog and of the pig is profusely supplied with blood by branches of the left and right coronary arteries and their many anastomoses. The atrioventricular node and the neighboring specialized conduction tissue thus receive blood from many sources. Prominent sinuses and venules in the atrioventricular node and a diffuse arrangement of arterioles and capillaries, as observed in the dog by Truex and Schwartz,[14] are also characteristic of the pig.

Our studies agree with the observations of others[10] regarding the extensive arterial blood supply to the interventricular septum of the dog. We have also demonstrated a far greater number of arteries going to the left ventricle than to the right in all species of domestic animals. Similarly, the left ventricle and interventricular septum possess a system of veins and sinuses that is much greater in complexity and capacity than that of the right ventricle. The venous system of the interventricular septum and left ventricle is more profuse than its arterial counterpart (fig. 6–9).

The complexity of the arterial supply to the interventricular septum in the dog and in the pig is contrary to the usual concept of the importance of the septal artery. The septal artery is not as significant in the nourishment of the cardiac conduction system as it was formerly thought to be; more oxygenated blood reaches the interventricular septum through the branches of the left descending artery than through the septal artery. In both species the interventricular branches from descending coronary arteries are so large that positive identification of septal arteries is difficult. Therefore, the interventricular septum is an important site of collateral arterial and venous circulation in the hearts of dogs and pigs.

Thebesian vessels, which connect coronary vessels with ventricular cavities, are capable of conducting blood in either direc-

tion, from the myocardium into the cavity or from the cavity into the myocardium, depending on the differential pressure gradient. In cases of coronary artery disease, the thebesian vessels may supply the myocardium with a small amount of blood.

Cineangiocardiographic studies of radiopaque solutions traversing the coronary vessels show the simultaneous filling of the major arteries during ventricular diastole under specific experimental conditions. When the sodium acetrizoate temporarily replaces oxygenated blood in the coronary arteries, a heart block may occur. The administration of oxygen during or just prior to the injection helps to relieve anoxia. If retrograde injections of sodium acetrizoate are given repeatedly via the right carotid artery, even at 30-minute intervals, tetanic convulsions, fibrillation, and subsequent death occur. During injection, blocking the opposite carotid artery with a rubber-covered hemostat prevents rapid passage of the solution to the brain, so these reactions do not occur. The slow passage of solution to the brain through the vertebral arteries causes no distress.

Cineangiocardiograph films, reduced to 16-mm. size, permit study of the filling of the major coronary arteries and the passage of the radiopaque solution through these arteries into the myocardial venous system. The finer details of coronary vasculature are not apparent when the sodium acetrizoate is disseminated in the myocardial sinuses. When the solution is again concentrated, just before entering the right atrium, the detailed structure and function of the coronary sinus is easily discernible (fig. 15).

Sinus reflux during atrial systole was demonstrated in 25 of 100 human cardiac patients by Campeti *et al.*[2] This was accompanied by reflux into the caudal vena cava during atrial diastole. The reflux of blood was primarily associated with hypertension and a failure of the coronary sinus valvular mechanism. The flow of sodium acetrizoate through the coronary sinus in the dog is shown (fig. 15). Sinus reflux has not been demonstrated in normal pigs and dogs.

The coronary veins have been outlined radiographically by retrograde injection of contrast material into a catheterized coronary sinus.[1, 12] The present work has the advantage of demonstrating the normal function of the sinus by passage of radiopaque material from the myocardium to the right atrium.

SUMMARY

The pattern of coronary arteries and veins in the dog and pig was described. Chloroprene latex and vinyl acetate casts, clearings, serial sections, and cineangiocardiographs were used to study the vasculature of 400 hearts.

The atrial vessels of the dog and pig differed in relative size and distribution of terminal branches, rather than in number or origin of major vessels. The blood supply to the interatrial septum and atrioventricular node was described The descending branch of the left coronary artery was more significant than the septal artery in supplying the interventricular septum.

Thebesian (luminal) vessels, which connect coronary vessels with ventricular cavities, were found in canine and porcine hearts.

Cineangiocardiographs helped to ascertain the functional pattern of blood flow in the principal coronary arteries and the coronary sinus. These studies supplemented data gained from casts of the detailed vasculature of the heart.

Distinct species differences occurred in hearts of dogs and pigs with respect to the distribution of atrial vessels, the terminal branches of the coronary arteries, and the modes of venous drainage.

References

[1] Aguzzi, A., di Guglielmo, L., Baldrighi, V., and Marley, A.: Visualizzazione del circolo venoso coronarico durante cardiangiografia. Radiol. med., *40*, (1954): 140.

[2] Campeti, F., Gramiak, R., Watson, J. S., Ramsey, G. H., and Weinberg, S.: Visualization of the Coronary Sinus in Cineangiocardiography. Circulation, *12*, (1955): 199–206.

[3] Christensen, G. C.: Circulation of Blood Through the Canine Kidney. Am. J. Vet. Res., *13*, (April, 1952): 236–245.

[4] Christensen, G. C.: Angioarchitecture of the Canine Penis and the Process of Erection. Am. J. Anat., *95*, (1954): 227–262.

[5] Christensen, G. C.: Comparative Architecture of Coronary Vessels and Cineangiocardiographic Studies in Domesticated Animals. Anat. Rec., *130*, (1958): 285.

[6] Gramiak, R., Watson, J. S., Ramsey, G. H., and Weinberg, S.: Cineangiocardiography in Congenital Heart Disease. A Study of 100 Consecutive Cases. New York J. Med., *53*, (1953): 1761.

[7] Halpern, M. H.: Arterial Supply to the Nodal Tissue in the Dog Heart. Circulation, *9*, (1954): 547–554.

[8] Halpern, M. H.: Blood Supply to the Atrioventricular System of the Dog. Anat. Rec., *121*, (1955): 753–762.

[9] James, T. N., and Burch, G. E.: Blood Supply of the Human Interventricular Septum. Circulation, *17*, (1958): 391–396.

[10] Moore, R.: The Coronary Arteries of the Dog. Am. Heart J., *5*, (1930): 743–749.

XUM

[11] Roberts, J. T.: The Role of the Small Vessels and Nerves of the Heart in Heart Failure, Coronary Artery Thrombosis and Cardiac Pain. M. Ann. District of Columbia, *14*, (1945): 483–496.

[12] Tori, G.: Radiological Visualization of the Coronary Sinus and Coronary Veins. Acta radiol., *36*, (1952): 405.

[13] Truex, R. C., and Angulo, A. W.: Comparative Study of the Arterial and Venous Systems of the Ventricular Myocardium with Special Reference to the Coronary Sinus. Anat. Rec., *113*, (1952): 467–492.

[14] Truex, R. C., and Schwartz, M. J.: Venous System of the Myocardium with Special Reference to the Conduction System. Circulation, *4*, (1951): 881–889.

[15] West, J. W., Kobayashi, T., and Guzman, S. U.: Coronary Artery Catheterization in the Intact Dog. Circulation Res., *6*, (1958): 383–388.

Isolation and Characterization of Cytopathogenic Enteroviruses from Cattle with Respiratory Disease

TORBJORN MOLL, D.V.M., Ph.D., and ADAMADIA D. DAVIS, M.S.

Pullman, Washington

DURING THE PAST DECADE a large number of cytopathogenic viruses have been found in the intestinal and upper respiratory tracts of man.[2,3] Various members of these "enteroviruses" and "adenoviruses" have been shown to be associated with transitory clinical syndromes in man, such as paralysis, aseptic meningitis, herpangina, myocarditis, encephalomyocarditis, summer diarrhea of infants and children, and undifferentiated febrile illnesses. These viruses also have been found in apparently normal individuals, but their incidence among healthy persons decreases with increasing age. Their etiological role in disease production has been established by demonstrating a higher prevalence and virus concentration among patients with these illnesses than in healthy individuals and by the development of specific antibodies during the course of illness.

A cytopathogenic virus has been isolated from the respiratory tracts of cattle with infectious bovine rhinotracheitis.[9] This virus has been adapted to human cells (HeLa) through alternating passage between HeLa cells and bovine embryo tissue cells.[1]

Moll and Finlayson[10] have reported on the isolation of a cytopathogenic virus from feces of cattle with a febrile respiratory illness and from the ascitic, stomach, and allantoic fluids from an aborted bovine fetus. Klein and Early[5] and Kunin and Minuse[6,7] have isolated cytopathogenic viruses from feces of apparently healthy cattle.

This is a report on the isolation and preliminary characterization of six cytopathogenic viruses from feces of cattle in six herds with histories of respiratory disease and abortion. These viruses have been designated "bovine enterovirus" (BE) in this laboratory.

From the Department of Veterinary Microbiology, College of Veterinary Medicine, State College of Washington, Pullman. Scientific paper No. 1726, Washington Agricultural Experiment Station, project No. 1144.

These investigations were aided by contract NR-103-325, with the Office of Naval Research.

MATERIALS AND METHODS

Cell Cultures.—Cell cultures were prepared from the kidney cortices of normal cattle, 6 to 24 months old, in 10- by 120-mm. tubes by the trypsin-digest method described by Younger.[12] The cells were grown at 37 C. in a nutrient medium consisting of Hanks' solution with 0.5 per cent lactalbumin hydrolysate and 10.0 per cent bovine serum or in Tris medium[8] with 10.0 per cent bovine serum.

When cell sheets were formed on the glass surface, the nutrient medium was withdrawn and replaced with a maintenance medium consisting of Hanks' solution with 0.5 per cent lactalbumin hydrolysate or Tris medium without serum. To both nutrient and maintenance mediums were added 100 to 200 units of penicillin, 100 to 200 μg. of streptomycin, and 100 to 200 units of nystatin (Mycostatin*) per milliliter.

Fecal Samples and Nasal Washings.—Fecal samples and nasal washings were suspended in phosphate buffered saline solution or Tris medium, without serum, containing 500 units of penicillin and 500 μg. of streptomycin per milliliter. Approximately 10 Gm. of fecal material was suspended in 25 ml. of medium. The suspensions were strained through a four-layer pad of cheesecloth into stainless steel tubes which were centrifuged at 10,000 r.p.m. for 30 minutes in a refrigerated centrifuge.

Each supernatant fluid was subsequently diluted 1:2 with maintenance medium and dispensed in 1-ml. quantities to each of three to five cell culture tubes. After two hours, this inoculum was withdrawn and replaced with fresh maintenance medium. Fetal fluids which were clear and free from bacteria were diluted 1:5 with maintenance medium and dispensed in 1-ml. quantities to each of five cell culture tubes.

Virus Assays.—Tenfold dilutions of virus-containing material were made in medium, using a fresh pipette for each dilution. Each virus dilution was inoculated into four cell culture tubes, 0.1 ml. per tube.

Serological Procedures.—Immune rabbit serums were produced against each BE virus. Two intravenous injections of 2 ml. each of infected cell culture fluid containing 10^6 to $10^{7.6}$ $t.c.d._{50}$ per milliliter were given to each 4-month-old rabbit at ten- to 14-day intervals. The serums were collected 14 days after the second virus injection. Rabbit and bovine serums were stored at −20 C.

* Mycostatin is produced by E. R. Squibb and Sons, New York, N.Y.

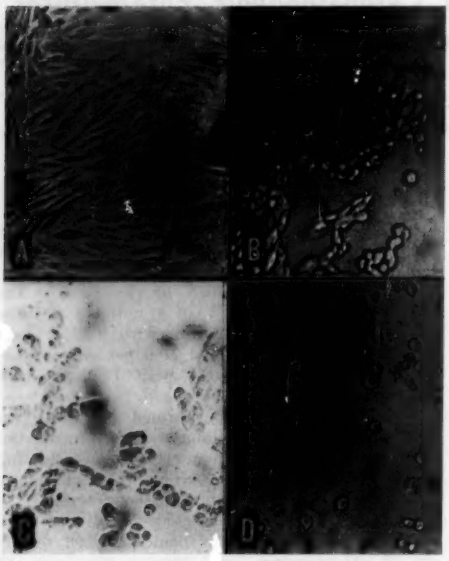


Fig. 1—Bovine kidney cell cultures: (A) normal cells growing on the glass wall of a test tube; (B) early cytopathogenic effect of virus growth—a number of cells have become shrunken or rounded, but most of the cell sheet appears normal; (C) progression of virus-induced cytopathogenic changes—a large number of cells are rounded or shrunken; (D) final stage of cytopathogenic changes—no normal cells remain, and most have fallen off the glass surface. x 15.

Neutralizing antibody titers were determined in cell culture-neutralization tests according to the method described by Paul and Melnick for paired poliomyelitis serums,[11] using fivefold dilutions of serums and constant amounts of virus. The log t.c.d.$_{50}$ was calculated by the method of Reed and Muench.

RESULTS

Primary Virus Isolations.—All six viruses were isolated from feces on first tissue culture passage. Virus could not be isolated from nasal or eye washings.

Virus BE-1.—This virus was recovered from the feces of 9 calves, 3 to 6 months old, and 2 pregnant cows, which were purchased and brought into an experimental barn unit. These animals developed fever (103–106 F.), slight cough, serous or mucous nasal exudate, and mild enteritis in seven to ten days. This respiratory disease syndrome was clinically diagnosed as "mild" or "abortive" shipping fever.

The affected calves recovered uneventfully within a week, but several had intermittent temperature elevations during the next three weeks. One cow (cow 230) also recovered in a few days, but aborted approximately one and one half months later. The aborted 6- to 7-month-old fetus showed marked serosanguineous subcutaneous and intermuscular edema and considerable amounts of serosanguineous fluid in the serous cavities. Virus was isolated from ascitic, stomach, and allantoic fluids. The other cow (cow 237) developed pulmonary complications and was treated with broad-spectrum antibiotics over a period of three weeks. When examined three months later, this cow was not pregnant.

Vibrio fetus, Leptospira pomona, and *Brucella abortus* were not found in the aborted fetus or in either cow.*

Feces from 9 of the animals were examined for the presence of virus at various intervals after the remission of clinical signs. All animals were found to shed virus up to three months after recovery. Five animals were shedding virus after six months, 3 after 12 months, and 1 after 15 months.

Virus BE-122.—This virus was isolated from a large beef herd in Montana. The herd had experienced considerable yearly losses from shipping fever among calves at weaning time (October, November).

The virus was isolated from four of six

* This information was made available by Dr. S. G. Kenzy, Department of Veterinary Microbiology, and Dr. D. M. Fluharty, Department of Veterinary Pathology, State College of Washington, Pullman.

TABLE 1—Log t.c.d.$_{50}$ Neutralizing Titers of Antiserums Against Homologous and Heterologous Viruses

Antiserums	Viruses					
	BE-1	BE-122	BE-160	BE-165	BE-167	BE-180
BE-1	(2.0)*	1.8	0.6	1.7	2.2	<0.6
BE-122	2.0	(1.7)	0.6	2.4	1.9	0.6
BE-160	2.2	2.0	(1.5)	2.4	1.8	1.7
BE-165	1.8	2.0	0.6	(3.1)	1.8	1.0
BE-167	2.4	3.0	0.6	1.8	(2.0)	<0.6
BE-180	2.7	2.7	2.5	3.0	2.5	(2.4)

* Numbers in parentheses represent homologous titers.

TABLE 2—Log t.c.d.$_{50}$ Neutralizing Titers of Known Human and Bovine Immune and Convalescent Serums Against Two Serological Types of BE Virus

Viruses	Serums									
	Polio I	Polio II	Polio III	IBR * Y-23	IBR Y-69	Agent X	Ulcer. stom.† 172-C39	Mucosal disease MD-48	Virus diarrhea VD-C9	ECBO **
BE-1	0.0	0.0	0.0	1.2	1.2	0.0	<0.6	<0.6	0.0	<2.7
BE-180	0.0	0.0	0.0	1.3	1.7	0.0	<0.6	<0.6	0.0	1.5

* IBR = infectious bovine rhinotracheitis; † ulcer stom. = infectious bovine ulcerative stomatitis; ** ECBO = enteric cythopathogenic bovine orphan.

fecal samples collected† from apparently normal calves in September and from 10 of 19 calves with clinical shipping fever at weaning time.

Virus BE-160.—The virus was recovered in December, 1957, from the feces of 5 calves, 6 months old, in central Washington. These calves were among about 100 which had fever, swelling of the submandibular region, excess nasal exudate, and mucus in feces.

Virus BE-165.—The virus was recovered from a dairy herd, in central Washington, which had a history of abortion. Several cows showed signs of anterior respiratory disease in November, 1957, and 6 cows aborted approximately one month later. The virus was isolated from feces of 4 of the 6 cows 14 days after abortion. The aborted fetuses were not available for study.

Virus BE-167.—This virus was recovered from a large beef cattle herd in southwestern Washington. This herd had experienced considerable losses from pinkeye, shipping fever, and occasional abortions. The virus was isolated from the feces of 3 of 6 cows which had pinkeye and later aborted. Aborted fetuses were not available for study.

Virus BE-180.—This virus was recovered from a small herd of beef cattle in eastern Washington. This herd had experienced some respiratory disease among the calves. The virus was isolated from feces of 3 of 4 calves which had been coughing.

Characterization of the Viruses Isolated.—All viruses isolated were cytopathogenic for bovine kidney cells grown *in vitro* (fig. 1). The cytopathogenic changes were characterized by focal rounding and shrinking of cells, which progressed until all cells were affected and fell off the glass surface. The fluid of the culture tubes in which the majority of cells were affected also was considerably more acid than the fluid of unaffected control tubes.

† By Dr. J. Palotay, who studied the disease outbreak.

Ten serial passages of infected cell culture fluids carried out have regularly resulted in cytopathogenic cell changes, as well as in increased acidity of the culture fluids.

Replicate titrations of various tissue culture passage levels showed a t.c.d.$_{50}$ of 10^5 to 10^9 per milliliter. Cell cultures inoculated with the lowest dilution of infected fluids showed typical cytopathogenic changes as early as four hours after inoculation; those inoculated with the highest dilution showed such changes 36 to 42 hours after inoculation.

The increase in acidity at the time of severe or complete cell destruction varied from 0.2 to 0.4 pH units as compared with normal cell cultures. Viral multiplication and cytopathogenic changes in bovine kidney cell cultures occurred at pH's of 6.4 to 8.4. The cytopathogenic changes developed more slowly and seemed less extensive at pH's of 6.4 to 6.6 than at pH's of 7.4 to 8.4. Considerable rounding and degeneration were observed in noninfected cultures at a pH of 8.4. The virus strains recovered from the six sources behaved identically in these trials.

Virus BE-1 produced cytopathogenic changes in swine kidney cell cultures. These changes were similar to those seen in the bovine kidney cell cultures. This virus strain also produced cytopathogenic changes in human amnion and kidney tissues, HeLa cell cultures, and *rhesus* and *patas* monkey kidney cells. Small irregular plaques were obtained on *rhesus* mono-

TABLE 3—Log t.c.d.$_{50}$ Titers of Convalescent Bovine Serums Against Virus BE-1

Animal (No.)	Months after onset of clinical disease					
	0–½	2–3	4–5	6–8	11–12	15
214	.....	0.7	3.0	1.7	3.4 *	3.4
244	0.4 *	0.0 *	1.8	2.5	1.5	....
248	1.6	2.5	1.7	1.8	1.7	....
250	0.2 *	2.2 *	3.2	2.5 *	2.3	....
251	0.2 *	1.0 *	1.3	2.4	1.1	....
230	.....	0.2	1.1 *	1.7	....	....
237	.....	0.6	1.0 *	1.7 *	....	....

* Virus isolated from feces.

layer cultures five to six days after inoculation. The plaque pattern resembled those of the enteric cytopathogenic human orphan (ECHO) viruses.** The other viruses isolated have not been studied in swine, monkey, and human cell cultures.

All six isolated viruses passed through an ultrafine glass filter without appreciable loss of titer. They also passed through an ultra-filter membrane with an average pore size of between 0.05 and 0.08 μ, but not through a membrane with an average pore size of 0.01 to 0.05 μ.

These viruses are ether-resistant. They survive storage in tissue culture maintenance fluid (pH 7.6–7.8) at −10 to −20 C. for at least one year, at 4 to 22 C. for at least two months, at 37 C. for at least 120 hours. and at 56 C. for at least 30 minutes.

Each isolated virus was tested against homologous and heterologous rabbit immune serums (table 1). From the data obtained, it appears that the six viruses fall into two serological groups; BE-1, BE-122, BE-165, and BE-167 belong to one group, and BE-160 and BE-180 to another. This indication was verified by statistical analysis using the Kendall rank correlation coefficient of concordance.[4] This analysis revealed a highly significant difference between the two groups but no significant difference between viruses within each group.

One virus of each serological group (BE-1, BE-180) was tested in tissue culture-neutralization tests against two infectious bovine rhinotracheitis (IBR) convalescent serums,* mucosal disease convalescent serum,† virus diarrhea convalescent serum,† infectious bovine ulcerative stomatitis convalescent serum,† bovine agent X convalescent serum,† enteric cytopathogenic bovine orphan (ECBO) virus rabbit immune serum,** and types I, II, and III monkey poliomyelitis immune serums‡ (table 2).

Both virus strains were neutralized to approximately the same degree by both IBR convalescent serums. Both strains also were neutralized by the ECBO immune serum, but one strain (BE-1) to a higher titer than the other. The other serums failed to neutralize either strain.

Disease-Producing Capacities.—Acute phase and convalescent serums collected from several animals in four of the six herds were tested for neutralizing antibodies against the virus isolated, using the tissue culture-neutralization test.

In one herd (table 3) all animals showed a rise in neutralizing antibodies after clinical recovery. Maximum neutralizing titers, which were higher in calves than in cows, occurred four to seven months after onset of clinical illness. Virus persisted in the intestinal tracts of several of these animals in spite of significant neutralizing titers.

Only one sample of convalescent serum was available from animals in the other herds. In each herd, several animals had significant titers against the virus two weeks to six months after recovery.

Experimental exposures of animals to these viruses are limited. Virus BE-1 caused neither signs of illness nor pathological tissue changes in normal suckling or weaned Swiss white mice when inoculated by the intraperitoneal, subcutaneous, or intranasal routes. However, this virus caused illness and extensive myocardial necrosis (fig. 2) in weaned mice when inoculated intraperi-

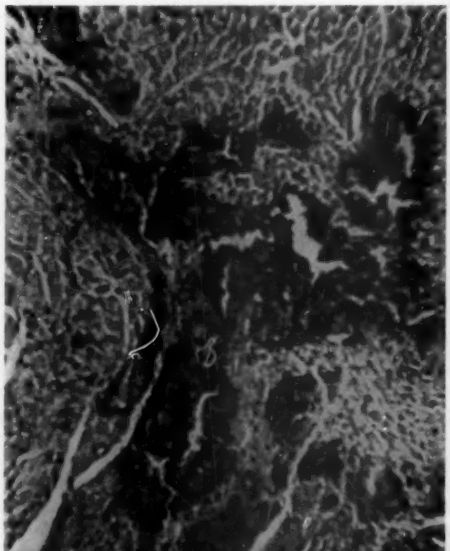


Fig. 2—Section of a mouse heart, showing areas of myocardial necrosis and calcification. x 100.

** This information was made available by Dr. G. D. Hsiung, Yale University School of Medicine, New Haven, Conn., and Dr. R. J. Huebner, National Institutes of Health, Bethesda, Md.

* Supplied by Dr. T. L. Chow, College of Veterinary Medicine, Colorado State University, Fort Collins; † Dr. W. R. Pritchard, Purdue University, Lafayette, Ind.; ** Dr. C. M. Kunin, Thorndike Memorial Laboratories, Boston, Mass.; ‡ Dr. C. A. Evans, School of Medicine, University of Washington, Seattle.

TABLE 4—Intracardial Inoculation of Pregnant Guinea Pigs with Virus BE-165 ($t.c.d._{50}$ Titer $10^{7.6}$/ml.)

Animal (No.)	Stage of pregnancy (days)	Amount of inoculum	Virus dilution	Termination of pregnancy
1	50–55	1 ml.	Undiluted	.....
2	45–50	1 ml.	Undiluted	3 stillborn offspring.
3	40–45	1 ml.	10^{-5}	6 stillborn offspring.
4	40–45	1 ml.	10^{-6}	4 stillborn offspring.
				1 living offspring; died 1 day later.
5	35–40	1 ml.	10^{-2}	2 stillborn offspring.
				2 living offspring; both died a few days later.
6	30–35	1 ml.	10^{-3}	4 living offspring; 1 died a few days later.
7	8–12	1 ml.	10^{-1}	4 living offspring; killed 1 day later.
8	40–45	1 ml.	Tissue culture fluid	4 living offspring; remained healthy.
9	45–50	1 ml.	Tissue culture fluid	4 living offspring; remained healthy.

torally after two to three daily intramuscular doses (2.5 mg.) of cortisone acetate.*

Various concentrations of virus BE-165 were inoculated intracardially into guinea pigs in various stages of pregnancy (table 4). Those inoculated after 40 days of pregnancy delivered stillborn offspring. Those inoculated before 30 days of pregnancy delivered living offspring. However, several of the living offspring appeared weak at birth and died later. Most of the dead fetuses showed serosanguineous subcutaneous and intermuscular edema, considerable amounts of serosanguineous fluid in the serous cavities, and marked fatty changes in the liver. The offspring which died after birth showed fatty degeneration of the liver. Virus could not be reisolated from organs of the dead guinea pigs.

Two pregnant guinea pigs which were inoculated intracardially with tissue culture fluid (Tris) during the last half of the gestation period delivered normal, healthy offspring.

Preliminary studies indicate that colostrum-fed calves, 2 to 10 days old, are not susceptible to virus BE-1 when exposed by the intraperitoneal, subcutaneous, intravenous, intranasal, and oral routes. The virus could not be reisolated from organs or feces after exposure.

Two calves, exposed to this virus by the intravenous and intraperitoneal routes when 1 month old, showed no clinical illness but had a few small areas of consolidation in the lungs when killed 14 days after exposure.

Two calves, 1 month old, which were exposed to this virus after three daily doses (250 mg.) of cortisone developed slight temperature elevation and nasal exudate, and showed large areas of grayish pink consolidations in the lungs when killed three weeks after exposure (fig. 3). The virus could not be reisolated from the lungs of these calves.

DISCUSSION

The isolation and characterization of six strains of bovine cytopathogenic enterovirus have been described. The cattle herds from which these viruses were isolated all had a history of respiratory disease or abortion previous to virus isolation.

The etiological relationship of the isolated virus with the clinical condition has not

Fig. 3—Calf lung, showing extensive areas of consolidation (right).

* Produced by Merck Sharp and Dohme, Inc., Philadelphia, Pa.

XUM

been established. Significant virus antibody titers and significant rise in such antibody titers following recovery tend to incriminate the isolated viruses as a causative factor.

Since the respiratory disease syndrome observed in cattle was mild and inconspicuous and the virus persisted in the intestinal tract for a long time after infection, isolation of these viruses from "normal" cattle would be anticipated. Futhermore, the extended period of persistence of the virus in the feces and the relatively high resistance of the viruses might well increase their spread to new premises and their maintenance on infected premises. The BE viruses might, therefore, prove to be common and widespread.

One of the BE viruses isolated caused marked myocardial necrosis in cortisone-treated mice. Preliminary experiments also indicate that this virus caused pneumonitis in cortisone-treated calves. Another virus, when inoculated into pregnant guinea pigs, caused stillbirths and weak offspring. These findings seem to indicate that the disease-producing capacities of the BE viruses are increased by stressing conditions.

Both serological types of BE virus were neutralized by antiserum against ECBO virus strains isolated by Kunin and Minuse [6,7] from feces of normal cattle. However, the latter viruses did not produce cytopathogenic changes in HeLa cells, and some strains caused paralysis of suckling mice.

Both serological types of BE virus were also neutralized by IBR convalescent serums. This may indicate serological relationship or, possibly, an experience of the cattle with BE virus. The serological relationship to IBR must be verified in cross-neutralization tests, using specific heterologous immune serums, such as rabbit serums. The respiratory disease syndromes observed in connection with the BE virus isolation did not resemble IBR. Virus could not be isolated from the respiratory tract, and the virus isolated from feces affected HeLa cells without any process of adaptation.

One of the viruses (BE-1) did not react in complement-fixation tests with human adenovirus antiserums. Its morphological effects in cell cultures resembled those of some of the ECHO viruses but its cell susceptibilities seemed different. In general, the bovine enteroviruses described appear to be unique in their broad spectra of cell susceptibility.

The incidence and distribution of these viruses among cattle herds, as well as their disease-producing capacities for cattle and laboratory animals and their relationship to other viruses, are being investigated further.

SUMMARY

The isolation and characterization of six strains of cytopathogenic bovine enterovirus have been described. These viruses were isolated from feces of cattle with histories of respiratory disease or abortion. All affected animals in one herd developed significant antibody titers after recovery.

The six viruses fall into two serological types. Both types were inhibited in tissue culture-neutralization tests by infectious bovine rhinotracheitis convalescent serums and enteric cytopathogenic bovine orphan virus immune serums. The cytopathogenic bovine enteroviruses described seem unique in their broad spectra of cell susceptibility, affecting bovine, porcine, monkey, and human cells (including HeLa cells) *in vitro* without any process of adaptation.

References

[1] Cabasso, V. J., Brown, R. G., and Cox, H. R.: Infectious Bovine Rhinotracheitis (IBR). I. Propagation of Virus in Cancer Cells of Human Origin HeLa. Proc. Soc. Exptl. Biol. & Med., *95*, (1957): 471–476.

[2] Committee on the Enteroviruses, National Foundation for Infantile Paralysis: The Enteroviruses. Am. J. Pub. Health, *47*, (1957): 1556–1566.

[3] Huebner, R. J., Rowe, W. P., Ward, T. G., Parrot, R. H., and Bell, J. A.: Adenoidal-Pharyngeal-Conjunctival Agents; A Newly Recognized Group of Common Viruses of the Respiratory System. New England J. Med., *251*, (1954): 1077–1086.

[4] Kendall, M. G.: Rank Correlation Methods. Charles Griffin and Co., Ltd., London, England, 1948.

[5] Klein, M., and Early, E.: The Isolation of Enteric Cytopathogenic Bovine Orphan (ECBO) Viruses from Calves. Bact. Proc. (1957): 73.

[6] Kunin, C. M., and Minuse, E.: The Isolation in Tissue Culture, Chick Embryo, and Suckling Mice of Filterable Agents from Healthy Dairy Cattle. J. Immunol., *80*, (1958): 1–11.

[7] Kunin, C. M., and Minuse, E.: New Bovine Viral Agents Isolated in Michigan. Pub. Health Rep., *72*, (1957): 251–252.

[8] Madin, S. H., Adriese, P. C., and Darby, N. B.: The *in Vitro* Cultivation of Tissues of Domestic and Laboratory Animals. Am. J. Vet. Res., *18*, (Oct., 1957): 932–941.

[9] Madin, S. H., York, C. J., and McKercher, D. G.: Isolation of the Infectious Bovine Rhinotracheitis Virus. Science, *124*, (1956): 721–722.

[10] Moll, T., and Finlayson, A. V.: Isolation of Cytopathogenic Viral Agent from Feces of Cattle. Science, *126*, (1957): 401–402.

[11] Paul, J. R., and Melnick, J. L.: Diagnostic Procedures for Virus and Rickettsial Diseases. 2nd ed. American Public Health Association Press, New York, N.Y. (1956): 53.

[12] Younger, S. J.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., *85*, (1954): 202–205.

Feline Infectious Anemia. II. Experimental Cases

JEAN C. FLINT, D.V.M., Ph.D.; MARTIN H. ROEPKE, Ph.D.;
RUE JENSEN, D.V.M., Ph.D.

Fort Collins, Colorado, and St. Paul, Minnesota

CLINICAL DATA on 30 cats with naturally occurring cases of feline infectious anemia have been outlined in the first paper of this series.[4] Experimental study of the disease was made through intravenous, intraperitoneal, and oral inoculation of 76 cats and intraperitoneal inoculation of 2 white rats, 26 white mice, 14 brown deer mice, and 2 dogs.

MATERIALS AND METHODS

Cats were obtained from city and county dog pounds at Salt Lake City, Utah. Consequently, their histories were unknown and their ages could only be estimated. Of the 76 cats, 30 were males and 46 were females; all were mature animals. Rats and mice were obtained from commercial sources. The 2 dogs were obtained from a breeder; they were 6 months old and had recovered from distemper.

All animals were kept under strict sanitary conditions and fed according to standard methods. They were isolated for a two-week period before experimental work began, during which time clinical examinations, temperatures, and blood counts were made at two- and three-day intervals. All animals were identified by ear tags or ear notching.

Hemoglobin determinations were made with a Spencer hemoglobinometer. Other hematological techniques were carried out according to methods outlined by Cartwright.[2]

Splenectomy of Experimental Animals.—After periods of observation of not less than three weeks, 23 of the cats, both dogs, and most of the rodents were splenectomized. Pentobarbital sodium (Nembutal *) anesthesia was used for the cats and dogs, and ether for the rodents. Sterile techniques were carried out in all operations.

Transmission Studies.—Transmission studies with cats were carried out with whole blood. Blood from cats with proved cases of infectious anemia was either citrated or heparinized to prevent coagulation and then injected as quickly as possible into experimental animals. The intraperitoneal, intravenous, and oral routes of inoculation were used with cats. Intraperitoneal inoculations were made to one side of the median line at a point about 2 inches anterior to the pubis. The bladder was expressed carefully before inoculation. Intravenous inoculations were made into the anterior cephalic vein. Oral inoculation was performed by placing varying amounts of blood in No. 000 gelatin capsules and then forcing them over the base of the tongue with forceps.

A total of 76 cats was experimentally inoculated with infective blood, of which 56 developed clinical and hematological signs of infectious anemia. Of these 56 cats, 23 were splenectomized and 33 were nonsplenectomized. Twenty cats either failed to develop recognizable signs of infection or died of other causes.

Amounts of infective blood used varied, and these data for all animals will be given in the section on results.

Eight cats were reinjected with infective blood at varying periods after they apparently had recovered from an initial experimental infection with *Haemobartonella felis.*

Four splenectomized cats were injected with pooled blood from cats which appeared normal clinically and hematologically.

Two splenectomized dogs were each inoculated intraperitoneally with 4.0 ml. of infective cat blood and, 23 days later, each was inoculated intraperitoneally with 10.0 ml. of infective blood from a different cat with infectious anemia.

Two white rats were splenectomized and held for 21 days to be sure they were not carriers of *Haemobartonella muris.* Each was then inoculated intraperitoneally with 1.0 ml. of infective cat blood. Rat 1 was given blood from an experimentally infected cat; rat 2 was given blood from a cat with a clinical infection.

Twenty-six white mice were splenectomized and held for 21 days. Each then was inoculated intraperitoneally with 0.5 ml. of infective blood from cats with both clinical and experimental cases.

Eight splenectomized brown deer mice were given 1.0 ml. of infected blood from an experimental cat and, 30 days later, were given 0.5 ml. of infected blood from a cat with a clinical infection.

Six splenectomized brown deer mice and 10 intact white mice were fed grain on which infected blood had been poured at the rate of 10 ml. of blood to 2 oz. of barley. They were kept on this ration until it had been consumed entirely (approx. 7 days).

Animals that died were necropsied for gross and histopathological studies.

From the College of Veterinary Medicine and Agricultural Experiment Station, Colorado State University, Fort Collins (Flint and Jensen), and the School of Veterinary Medicine, University of Minnesota, St. Paul (Roepke).

A portion of these data was included in a thesis submitted by the senior author to the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the Ph.D. degree.

* Nembutal is produced by Abbott Laboratories, North Chicago, Ill.

XUM

TABLE 1—Relapses in Two Cats After Splenectomy

Item	Cat 19	Cat 51	Mean
Days between apparent recovery and splenectomy	7	60	33.5
Average hemoglobin level before splenectomy (Gm./100 ml.)	7.50	10.50	9.0
Days until blood smears positive after splenectomy	6	28	17
Hemoglobin level on first day positive (Gm./100 ml.)	6.5	8.0	7.25
Lowest hemoglobin level after splenectomy (Gm./100 ml.)	4.5	8.0	6.25
Day of lowest hemoglobin level after splenectomy	10	28	19
Peak temperature (F.)	105.4	103.0	104.2
Day of peak temperature after splenectomy	9	56	31.6
Days until death after splenectomy	12	56*	.....

* Animal was killed.

Treatment of various types was given to 19 cats with positive cases of feline infectious anemia. Blood transfusions, arsenical injections, and antibiotic therapy were used.

RESULTS

Feline infectious anemia was produced experimentally in both nonsplenectomized and splenectomized cats of varying ages by the introduction of infected blood orally, intraperitoneally, and intravenously. Experimental cases could not be differentiated clinically, hematologically, or pathologically from spontaneous cases, when comparisons were made during the acute stage. Cats that recovered from the acute stage of feline infectious anemia were not immune to subsequent attacks when reinjected with infected blood.

Of 76 cats given inoculations with infected blood or other infected material by various routes, 56 developed the disease (table 4), as indicated by positive blood smears. Of these, 33 were nonsplenectomized and 23 were splenectomized.

Summary of Transmission Studies Using Nonsplenectomized Cats—Intraperitoneal Inoculation of Infected Blood.—Of 21 cats inoculated intraperitoneally, 20 had positive blood smears at periods of 9.0 to 34.0 days, with an average of 15.7 days. Blood smears from 1 typically infected cat were negative for organisms, but the hemoglobin level dropped to 4.0 Gm./100 ml. on day 20.

Of the 21 cats, 18 showed hemoglobin levels of 6.5 Gm./100 ml. or less during the course of their infections; the average time to reach the low level was 20 days. Three cats showed positive blood smears, but their hemoglobin levels did not drop below 9.0 Gm./100 ml.

Intravenous Inoculation with Infected Blood.—Of 10 cats, 8 had positive blood smears at periods of 8.0 to 23.0 days, with an average of 16.4 days. Two cats failed to show the organisms in peripheral blood smears, although they became anemic (Hb, 6.0 and 4.0 Gm./100 ml., respectively) and had other typical signs of the disease. Peak temperatures ranged from 102.0 to 105.2 F., with an average of 103.6 F.

Oral Inoculation with Infected Blood.—Two cats were given 2.0 and 5.0 ml., respectively, of infected blood. The first had positive blood smears on postinoculation day 31 and died on day 43; the second had positive blood smears on day 13 and died on day 26.

Summary of Transmission Studies Using Splenectomized Cats—Intraperitoneal Inoculation with Infected Blood.—Nine cats inoculated by this route had positive blood smears at periods of 9.0 to 32.0 days, with an average of 16.8 days. Seven of them had hemoglobin levels of less than 6.5 Gm./100 ml. during the course of the infections. Four were killed during the height of the infection; the other 5 died at periods of 16 to 34 days.

Intravenous Inoculation with Infected Blood.—Ten cats inoculated by this route had positive blood smears at periods of 5.0 to 39.0 days, with an average of 13.3 days. Eight cats developed hemoglobin levels of 6.5 Gm./100 ml., or less.

Oral Inoculation with Infected Blood.—Four cats inoculated by this route had positive blood smears at periods of 22.0 and 51.0 days, with an average of 41.5 days. Only 1 had a hemoglobin level as low as 6.5 Gm./100 ml. However, temperatures were uniformly high and, clinically, the infection was typical. All 4 cats recovered.

Relapses in Two Cats After Splenectomy.—Two nonsplenectomized cats inoculated with infective blood showed positive blood smears and apparently recovered (cat 19 without treatment, cat 51 with treatment). After their hemoglobin levels had returned to normal, they were splenectomized. Within seven days after splenectomy, the hemoglobin level of cat 19 had dropped from 8.5 to 5.5 Gm./100 ml. and, on the tenth

XUM

TABLE 2—Data on Eight Cats Reinjected with Infected Blood After Apparent Recovery from Previous Attack

Cat No.	Spleen condition	Day reinjected after recovery	Hb when reinjected (Gm./100 ml.)	Route of injection	Amount blood (ml.)	Days until positive	Lowest Hb (Gm./100 ml.)	Peak temperature (F.)	Termination
12	Intact	60	10.0	i.p.	2.5	22	4.75	104.0	Recovered**
25	Intact	60	12.5	i.p.	2.0	23	8.50	103.0	Recovered
11	Splenectomized*	44	11.5	i.p.	2.5	....	6.00	104.0	Recovered
52	Splenectomized†	22	9.0	i.v.	5.0	7	5.00	103.0	Recovered
53	Splenectomized†	79	10.0	i.p.	2.0	17	4.00	105.0	Died (34)
55	Splenectomized†	27	9.5	i.v.	13.0	8	6.50	102.6	Recovered
56	Splenectomized†	72	10.5	i.p.	1.5	15	4.00	105.2	Died (24)
59	Splenectomized†	44	12.0	i.v.	1.0	16	5.50	103.8	Died (16)

* Cat was splenectomized prior to first inoculation and infection; † cat was splenectomized between original and second infection; ** cat recovered without treatment.

day, the hemoglobin level was 4.5 Gm./100 ml. It died on the twelfth day.

Cat 51 had a hemoglobin level of 12.0 Gm./100 ml. when splenectomized and had been normal, clinically and hematologically, for 72 days. On day 28 following splenectomy, the hemoglobin level was 8.0 Gm./100 ml., and the blood smear showed numerous parasites on the erythrocytes. During the next 25 days, the cat had only three positive blood smears. When killed on day 57, its hemoglobin level was 9.5 Gm./100 ml. The leukocyte count never was less than 31,000 per cubic millimeter of blood during this period, and rose to 49,600 before death. Detailed data on these 2 cats are given (table 1).

Reinjection of 8 Cats with Infective Blood After Apparent Recovery from an Earlier Infection.—The cats had appeared normal, clinically and hematologically, for periods of 22 to 79 days when they were reinjected. Two of them were intact, 1 was splenectomized before the initial inoculation, and 5 were splenectomized between the first and second inoculations with infective blood. Seven had positive blood smears between days 7 and 23, 3 died, and 5 recovered without treatment (table 2).

Transmission Studies Using Blood from Apparently Normal Cats.—Four splenectomized cats were inoculated with pooled blood from hematologically normal cats that had entered the clinic for treatment of cat bite abscesses or other minor ailments (table 3). All 4 had positive blood smears within the prescribed periods of time; 3 developed severe anemias, with hemoglobin levels of 6.5 Gm./100 ml. or less. Three cats recovered without treatment and 1 died.

Summary of Transmission Studies Using Rats, Mice, and Dogs.—In no instance was it possible to show infection in these animals, as indicated by positive blood smears, anemia, or death.

Results of Hematological Studies in 56 Experimental Cats—Hemoglobin.—Of 690 hemoglobin determinations made during preinoculation periods, the average value was 11.34 Gm./100 ml. After infection could be shown by Giemsa-stained smears, 518 determinations showed an average hemoglobin content of 6.41 Gm./100 ml.

Erythrocytes.—Similarly, 463 counts made before inoculations gave an average of 7,940,000 erythrocytes per cubic millimeter of blood. In cats showing infection, the average was 3,300,000 per cubic millimeter.

Leukocytes.—The average of 589 counts made before inoculation with infective blood was 19,980 leukocytes per cubic millimeter of blood. In 337 counts on infected cats, it was found to be 18,060 leukocytes per cubic millimeter.

Hematocrit Determinations.—A total of 144 determinations for packed erythrocyte volume was made on 27 experimental cats before inoculation with infective blood. The

TABLE 3—Injection of Four Splenectomized Cats with Blood from Apparently Healthy Cats Selected at Random

Item	Cat 55	Cat 59	Cat 61	Cat 67	Mean
Route of inoculation	i.v.	i.v.	i.p.	i.v.	.....
Amount of blood used (ml.)	13.0	4.0	5.0	4.0	6.5
Days until blood smears positive	5	16	32	39	23
Days until anemic	8	16	....	39	21
Hemoglobin level of first day positive (Gm./100 ml.)	8.5	6.5	8.5	5.0	7.1
Lowest hemoglobin level (Gm./100 ml.)	6.5	4.0	7.5	5.0	5.75
Peak temperature (F.)	102.6	103.8	102.4	105.4	103.5
Day killed	61*	52	32*	52*	....

* Animal was recovering when killed.

XUM

average was 34.96 per cent. After infection was established and the hemoglobin levels had dropped to 7.5 Gm./100 ml. or less, 56 hematocrit determinations made on 13 cats showed the packed erythrocyte volume to be 17.97 per cent, or 51.1 per cent of the normal hematocrit value.

Sedimentation Rates for One Hour.—A total of 136 determinations of sedimentation rates on 21 experimental cats before inoculation gave an average rate of fall of 42.9 mm. per hour. After infection was established, 56 determinations on 13 cats gave a sedimentation rate of 69.89 mm. per hour. No correction was made in the latter data for increased rate due to a reduction in total number of erythrocytes.

Mean Corpuscular Volumes of Erythrocytes.—A total of 144 determinations on 27 experimental cats before inoculation with infected blood gave an average mean corpuscular volume of 46.73 $c\mu$. After infection was established, 56 determinations on 13 cats gave an average mean corpuscular volume of 58.37 $c\mu$.

Mean Corpuscular Hemoglobin.—A total of 144 determinations on 27 experimental cats before inoculation with infected blood gave an average mean corpuscular hemoglobin level of 15.33 $\mu\mu g.(\gamma\gamma)$. On 13 infected cats, the average was 18.00 $\gamma\gamma$ in 56 determinations.

Mean Corpuscular Hemoglobin Concentration.—A total of 144 determinations on 27 experimental cats before inoculation with infected blood gave an average mean corpuscular hemoglobin concentration of 32.83 per cent. In 13 infected cats, 56 determinations gave an average of 31.05 per cent.

Gross and Histopathological Studies.—Minimal lesions were present at necropsy of 56 cats experimentally infected with feline infectious anemia. Aside from the marked anemia which was present consistently, icterus, enlargement of the spleen and, to a lesser extent, enlargement of lymph nodes were common lesions (Table 5).

Histopathological examination of tissues revealed little additional information. Sinusoids of the spleen were enlarged, with erythrocytes and large amount of blood pigment present in macrophages. Lymph nodes were hemorrhagic in about 25 per cent of the cats and edematous in a slightly higher percentage. Hyperplasia of the bone marrow was evident in most cats which were not in final stages of the disease. Other organs, on the whole, were free of lesions.

Treatment of Experimentally Infected Cats.—Twenty blood transfusions were given to 14 cats experimentally infected with infectious anemia; ten responses were considered good, six fair, and four poor. Hemoglobin levels of most of the cats were below 5 Gm./100 ml. when transfusions were given; some of these cats were nearly comatose. Approximately 8.0 ml. per pound of body weight (40 ml. for the average-sized cat) gave best results. Repeated transfusions, at intervals of three or four days, proved beneficial in most instances.

Oxyphenarsine hydrochloride (Mepharsen*), an arsenical compound, was used in treating 10 cats experimentally infected with feline infectious anemia during acute stages of the disease. Six made good recoveries, 3 made fair responses, and 1 failed to show improvement. Cats vary in their reactions to oxyphenarsine hydrochloride. In general, the reactions tend to be severe,

* Mepharsen is produced by Parke, Davis and Co., Detroit, Mich.

TABLE 4—Summary of Transmission Studies (Mean Figures for 56 Cats)

Methods of transmission	Spleen* condition	Days until positive	Days until anemic	Day of lowest Hb	Peak temp. (F.)	Day of peak temperature
Intraperitoneal	Nonsplenectomized (21)	15.70	20.00	23.10	103.9	15.55
	Splenectomized (9)	16.77	20.14	18.66	104.0	16.7
Intravenous	Nonsplenectomized (10)	16.40	18.90	21.10	103.6	16.50
	Splenectomized (10)	13.30	19.25	24.30	103.9	15.90
Parenteral mean		15.53	19.82	21.79	103.85	16.16
Oral	Nonsplenectomized (2)	22.00	29.50	33.50	104.5	18.00
	Splenectomized (4)	41.50	.....	56.50	104.3	36.50
Oral mean		31.75	.....	45.00	104.4	27.25

* Number of cats shown in parentheses.

with vomiting and diarrhea as frequent sequelae.

Antibiotics were used in treating 11 experimentally infected cats. Five were given oxytetracyline (Terramycin†) orally, at the rate of 100 mg. each morning and night. In 2 cats, where the treatment was continued for 20 days, good responses were observed; in 1, slight improvement occurred after eight days of treatment. Two cats showed no favorable responses to treatment.

Tetracycline (Achromycin, Tetracyn **) gave good results orally, at the same dosage and schedule, in all 6 cats in which it was used. In each instance, treatment was continued for 18 to 29 days.

DISCUSSION

As reported in the first section of this study, feline infectious anemia appears to be widespread in the United States. The number of diagnosed cases increases as practitioners become aware of the clinical symptoms and look for the disease.

Experimentally, the disease resembles hemobartonellosis in other animals in many respects, but with this outstanding difference: The effect of splenectomy is minor in cats, whereas it is of prime importance in rodents, dogs, and other animals.[8]

There were only minor differences between splenectomized and nonsplenectomized cats, regardless of the type of parenteral infection. When inoculated orally, it took almost twice as long, on the average, for splenectomized cats to show positive reactions, but the numbers were too small in this instance to be conclusive (2 nonsplenectomized, 4 splenectomized; table 4).

However, (table 1), 2 intact cats which apparently had recovered from experimental infections relapsed with acute cases within 6 and 28 days, respectively, after splenectomy. It is probable that any stress condition (such as pregnancy, starvation, hysterectomy) would have produced a relapse. It is believed that most spontaneous cases seen in practice are relapses in cats that have experienced stress conditions. A number of the severely infected experimental cats in this study made satisfactory clinical recoveries without treatment, and it is believed that this occurs frequently in cats at home.

TABLE 5—Gross Findings at Necropsy of 56 Infected Cats

Lesions	No. cats
Generalized anemia	56
Emaciation	34
Icterus	43
Splenomegaly	37
Mesenteric lymphadenitis	26
Perpheral lymphadenitis	17
Pericarditis	5
Cystitis	7
Pneumonia	1
Bone marrow hyperplasia	54
Gastroenteritis	3
Internal parasites	7*

* Six cats were infected with roundworms, 1 with tapeworms.

Many older cats apparently carry the infective organisms for long periods of time (table 3). Flint and Moss[3] and Splitter *et al.*[6] confirm this. It is apparent that donor cats constitute a hazard to routine blood transfusions in the clinic.

IMMUNITY

There is no evidence to indicate that the cat acquires an active immunity to feline infectious anemia. A study of table 2, which gives data on 8 cats which were reinjected with infective blood after apparent recovery from initial attacks, indicates that any immunity which is established is weak or of short duration, or both. It may be similar to pneumonitis in cats in this respect.

It would appear that premunition is the only type of immunity existing in this disease. Neitz,[5] who studied Bartonella and eperythrozoa extensively, stated:

> It is difficult to explain why relatively few animals develop clinical symptoms after infection (with Haemobartonella). In *Haemobartonella muris* infection in rats, *Trypanosoma gambiense* may interrupt the premunity, and in *Eperythrozoon coccoides* infection in mice, *Plasmodium berghei* may cause a relapse. *Haemobartonella canis* in Europe occurs as a submicroscopic infection while in India this organism can be demonstrated in large numbers in non-splenectomized dogs. *Eperythrozoon suis* has been encountered in your country in swine after immunization against hog cholera. Thus it appears that under stress of circumstances, relapses can develop.

It is reasonable to assume that large numbers of cats contract the disease during the first three years of their lives. Most of the cases are subclinical and are not diag-

† Terramycin is produced by Chas. Pfizer and Co., Brooklyn, N.Y.

** Achromycin is produced by Lederle Laboratories, Pearl River, N.Y.; Tetracyn is produced by Chas. Pfizer and Co., Brooklyn, N.Y.

XUM

nosed. Recovery probably is slow but uneventful in most instances, with only a few cases becoming acute.

It is believed that stress conditions, such as starvation, cold, pregnancy, neoplasia, abscesses, pneumonitis, and other factors of this type, lower resistance and allow the Haemobartonella organisms to multiply and produce clinical signs of the disease. Each of these stress factors has been encountered in cats with clinical cases in Salt Lake City. This accounts for the high percentage of cats with "relapsed" cases, often chronic in nature, which are seen in veterinary clinics.

At this stage of the investigation, there is virtually no evidence to indicate how the infection is spread. In most Haemobartonella infections of other animals, it has been assumed that insect vectors are the most common means of spread. However, in Salt Lake City, fleas are virtually unknown and mosquitoes and other insects are uncommon within the city, so it is not likely that insect vectors are mainly responsible there.

Infected and noninfected cats have been kept in the same cages for months at a time without evidence of spread of the infection.

Aside from blood smears which show the organisms, the signs of feline infectious anemia are nonspecific and, unfortunately, the organisms are discernible in peripheral blood smears only part of the time. However, hemoglobin determinations and leukocyte counts are of major importance in diagnosis. The former give the most accurate measure of the degree of anemia and the latter indicate the body's response to the challenge of the invading organisms. In young cats, leukocyte counts also are of value in differentiating the disease from feline panleukopenia.

The average hemoglobin level for normal cats is about 11.0 Gm./100 ml., according to most workers. In this study, the average for 690 determinations on 56 normal cats was 11.34 Gm./100 ml. It was decided, arbitrarily, on the basis of clinical and experimental observations, that a hemoglobin level of 6.5 Gm./100 ml. would be considered the "breaking point" and that cats with lower levels would be considered anemic. In a majority of instances, cats having hemoglobin levels of 6.5 Gm./100 ml. or less showed *H. felis* organisms in peripheral blood smears at some time, and most underwent further lowering of hemoglobin levels.

Anemia in the cat was relatively uncommon in 20 years of clinical experience. While other factors may cause anemia, a possible diagnosis of feline infectious anemia should be considered when hemoglobin levels of 6.5 Gm./100 ml. or less occur.

Macrocytosis is another sign which is indicative of anemia and, possibly, feline infectious anemia. Increased size of erythrocytes is readily apparent in cats with advanced cases; as many as 50.0 per cent of the cells may be the size of canine erythrocytes. The mean corpuscular volume of normal feline erythrocytes was found to be 46.73 $c\mu$, while the average for infected animals was 58.37 $c\mu$. This increase of 27.05 per cent apparently is due to the forcing out of the bone marrow of many immature cells in an effort to meet the challenge for more oxygen-carrying capacity. When macrocytosis is present, anisocytosis necessarily is prominent and, usually, nucleated erythrocytes and large blue-staining normoblasts are numerous.

ETIOLOGY

The organisms, believed to be a species of the genus Haemobartonella, were described in the first section of this paper. The classification as Haemobartonella species is based on the following: (1) Similar organisms have been described in man (bartonellosis) and in rodents and dogs, and have been classified as Haemobartonella in "Bergey's Manual,"[1] and by Weinman[8] and others; and (2) the only other group of organisms which resembles those seen in the cat are eperythrozoa, which have been found in cattle, sheep, hogs, and rodents, and can be differentiated without great difficulty.[7] Eperythrozoa nearly always are widely dispersed in the plasma; Haemobartonella organisms rarely are seen free of the erythrocyte and, when so found, probably have just been released by a hemolyzed erythrocyte.

Further, eperythrozoa predominate as "ring" forms, although rod and coccoid forms are described. Neitz[5] examined blood smears from this series of clinical and experimental cases, and stated: "I have spent some time examining the slides and have come to the conclusion that the parasites belong to the genus Haemobartonella. In some respects this organism resembles *Haemobartonella canis* (Kikuth)."

In this connection, it should be recalled that recognizable infections did not occur in

2 splenectomized pups after injection of infective cat blood.

Therapy

Evaluation of therapeutic measures usually is difficult and, in this study, it was particularly so, as the disease processes were largely unknown. Under field conditions, it is probable that many cats recover without treatment, but remain latent carriers of the disease.

The earliest attempts at treatment were trial and error procedures, with poor results, on the whole. Many of the affected cats were moribund when presented at the clinic. Nevertheless, 8 of 15 treated cats did recover.[4] Of 19 experimentally infected cats treated, 15 recovered. This appears to indicate that proper treatment does increase the chance of recovery.

Blood transfusions are essential for the severely anemic animal. The cat may be kept alive by this method long enough to permit other therapeutic agents to become effective. A number of cats with both clinical and experimental infections made uneventful recoveries after two or more blood transfusions, without the aid of antibiotics. Unless the patient is moribund, the response to transfusion usually is excellent, for a day or two at least. Transfusions should always be the first therapeutic measure.

Arsenicals are presumed to be almost specific for hemobartonellosis in other animals, but the cat often is hypersensitive to them. In this study, they were discarded in favor of antibiotics. Arsenicals must be given intravenously and there is a wide range of toleration. In this study, severe diarrhea, depression, and vomiting often followed its use; these conditions were not conducive to recovery in animals already near death.

Oxytetracycline and, later, tetracycline hydrochloride were used effectively in conjunction with blood transfusions. Results were better when it became obvious that, as in anaplasmosis, treatment must be continued for at least 21 days.

Better methods of treating feline infectious anemia are needed, and further investigation should be directed along this line.

Fields for Further Study

This investigation necessarily was limited in scope, and the following matters were encountered which need further study:

1) Differential leukocyte analysis. There was considerable variation in the differential blood picture in this disease. Prognosis might be determined by a better understanding of the leukocyte changes.

2) Method of spread among cats under normal field conditions.

3) Methods of immunizing against feline infectious anemia.

4) Bone marrow studies. Preliminary investigations indicate that bone marrow smears may show organisms on immature erythrocytes when they are not present in peripheral blood.

5) Methods of culturing the organisms on artificial mediums.

6) Improved methods of diagnosis. Newer techniques need to be developed, with possible adaptation of those now used in the study of anaplasmosis.

7) Therapeutic studies. Only a few therapeutic agents were given a preliminary trial in this study. This number should be expanded in an effort to find an agent that will kill all the organisms within the cat's body.

Summary

1) Feline infectious anemia is an acute or chronic blood parasitic disease characterized by emaciation, depression, anorexia, and initial high temperatures. A macrocytic hemolytic anemia develops, which produces a characteristic hemogram.

2) Data are presented on 56 cats experimentally infected with the etiological agent of feline infectious anemia. These data supplement those reported previously for 30 cats with clinical cases of the disease. The name, *Haemobartonella felis*, is proposed for the organisms.

3) Cats of all ages are readily infected with the organisms by intraperitoneal, intravenous, or oral routes. The effect of splenectomy is minimal in the cat, in contrast to other animals.

4) Transmission of the infective agent to splenectomized rats, mice, and dogs was not successful.

5) Immunity to the disease is weak or transitory, or both. Relapses occur and cats that appear, clinically and hematologically, to have recovered from the disease may be reinfected by injections of the organisms.

6) Broad-spectrum antibiotics and blood

transfusions offer the best method of treatment at present.

References

[1] Breed, Robert S., Murray, E. G. D., and Smith, Nathan R.: Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins Co., Baltimore, Md., 1957.

[2] Cartwright, George W.: Diagnostic Laboratory Hematology. Grune and Stratton, New York, N.Y., 1954.

[3] Flint, Jean C., and Moss, Lloyd C.: Infectious Anemia in Cats. J.A.V.M.A., *122*, (Jan., 1953): 45–48.

[4] Flint, Jean C., Roepke, Martin H., and Jensen, Rue.: Feline Infectious Anemia. I. Clinical Aspects. Am. J. Vet. Res., *19*, (Jan., 1958): 164–168.

[5] Neitz, W. O; Director of Veterinary Services, Union of South Africa: Personal communication, 1955.

[6] Splitter, Earl J., Castro, Edin R., and Kanawyer, Wendell L.: Feline Infectious Anemia. Vet. Med., *51*, (1956): 17–22.

[7] Tyzzer, E. E.: Interference in Mixed Infections of Bartonella and Eperythrozoa in Mice. Am. J. Path., *17*, (1941): 141.

[8] Weinman, David: Infectious Anemias Due to Bartonella and Related Red Cell Parasites. Tr. Am. Phil. Soc., *33*, (1944): 243–351.

Observations on Pyometra in Dogs, with Reference to the Hypothalamic-Hypophysial Neurosecretory System

S. TALANTI, D.V.M.

Helsinki, Finland

PYOMETRA, or hyperplastic endometritis, is encountered most frequently in bitches over 5 years of age. It is characterized by an accumulation of pus within the uterus. The cause of this condition is unknown. The contents of the affected uterus often are sterile.[14] In some cases, however, there may be bacterial contamination.[1] This infection has been considered a causative factor. However, according to results presented by a number of authors, we would have to assume that hormone imbalance is an important primary etiological factor.[8, 14, 16]

Polydipsia often is the first sign observed. Polyuria also may be noticed. Simultaneously with polyuria, the specific gravity of the urine is low, in many cases approximately 1.001 to 1.005. The kidneys have lost, at least to some extent, the capacity to concentrate urine.[3] At a more advanced stage of the disease, this impairment of renal function can be due to organic renal changes. However, our preliminary observations showed that the kidneys are intact at the beginning of the disease.

Apparently, the principles of the posterior lobe of the hypophysis function as a regulator in the water balance of the organ. According to the theory of neurosecretion,[13] these principles of the posterior lobe are synthesized in the hypothalamic ganglion cells of the supraoptic and paraventricular nuclei. They migrate within the nerve fibers of the supraoptico-hypophysial and paraventriculo-hypophysial tract into the neurohypophysis. Here they are stored and, if necessary, released. Much hypothetical evidence has been presented to support the view that the principles of the posterior lobe are histologically demonstrable with some selective staining methods,[2] e.g., Gömöri's chrome-alum-hematoxylin-phloxine method[6] and Gömöri's aldehyde-fuchsin method.[5]

In some pathological conditions, changes in the amount of stainable neurosecretory substance can be demonstrated with these methods. Strong dehydration causes disappearance of the material to some extent.[12] It was observed that, in experimental diabetes insipidus, material was practically absent in the neurohypophysis.[9] As disturbances in water balance apparently exist in animals with pyometra, at least at an early stage, it can be assumed theoretically that changes also occur in the hypothalamic-hypophysial neurosecretory system.

The purpose of this study was to determine if it was possible to demonstrate these changes by using purely histological methods. The staining was done with Gömöri's aldehyde-fuchsin reagent, which has proved particularly applicable in demonstrating neurosecretory phenomenon.[4, 7, 10, 15]

MATERIALS AND METHODS

Six bitches, 6 to 11 years old, were killed because of clinically confirmed pyometra. They had marked polydipsia and were killed at an early stage of the disease. Necropsy showed that all had a distinct accumulation of pus within the uterus. No evidences

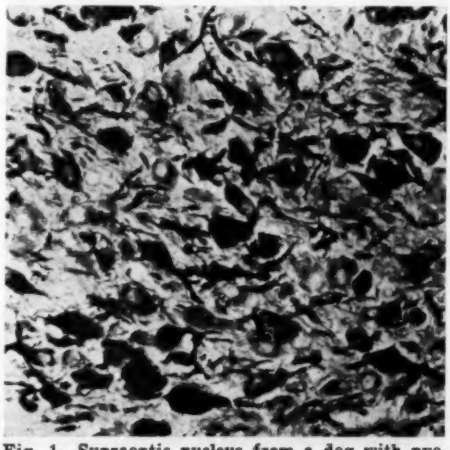


Fig. 1—Supraoptic nucleus from a dog with pyometra. Some ganglion cells are filled with strongly stainable aldehyde-fuchsin-positive material. Gömöri's aldehyde-fuchsin stain; x 400.

From the Department of Anatomy, Veterinary College, Helsinki, Finland.

This investigation was supported by a grant from the State Board for the Natural Sciences.

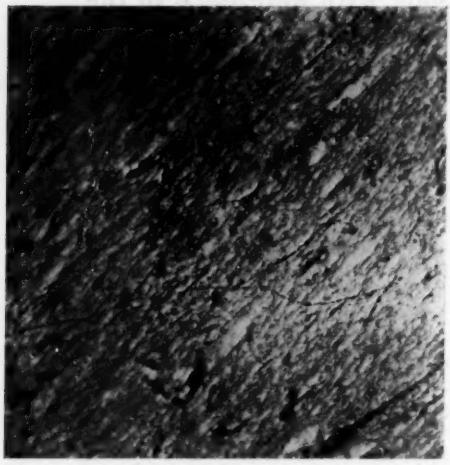


Fig. 2—Supraoptic-hypophysial tract from a dog with pyometra, showing rows of aldehyde-fuchsin-positive granules. Gömöri's aldehyde-fuchsin stain; x 200.

of pathological changes in the kidneys could be found.

The control group consisted of 10 bitches, 5 to 13 years old, which were demonstrated to be healthy by both clinical examinations and necropsies.

After the animals were killed, the brains were removed, avoiding postmortem injuries. The pieces of the brains were fixed in Bouin's fluid and embedded in paraffin. After deparaffinization and hydration through xylene and alcohol series, they were stained with Gömöri's aldehyde-fuchsin method as modified by Landing *et al.*[11] The sections from dogs in the control group were stained under the same conditions.

RESULTS

Supraoptic and Paraventricular Nuclei.—Strongly stainable granular substance occurred in the secretory ganglion cells of the dogs in the control group, as well as of the bitches with pyometra (fig. 1). The groups apparently did not differ much from each other, although the number of ganglia devoid of granules in bitches with pyometra appeared to be greater than that in dogs in the control group.

Supraoptico-Hypophysial and Paraventriculo-Hypophysial Tract.—Neurosecretory substance in dogs of both groups existed as granular material. The granules formed long rows along the length of the tract (fig. 2). Occasional accumulations with abundant aldehyde-fuchsin-positive material (so-called "Herring bodies") could be found. No differences between the groups could be observed.

Neurohypophysis.—Stainable material in bitches of the control group was seen as small granules distributed densely over the entire gland (fig. 4). Material was most abundant around the blood vessels. The amount of material in bitches with pyometra was conspicuously smaller. In some

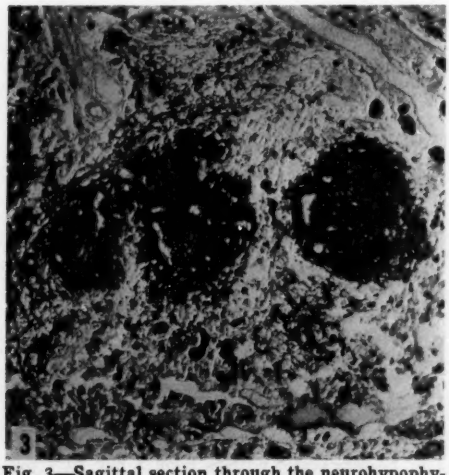


Fig. 3—Sagittal section through the neurohypophysis of a dog with pyometra. The distribution of the neurosecretory material is heterogeneous. A zone of the adenohypophysis is shown at bottom. Gömöri's aldehyde-fuchsin stain; x 180.

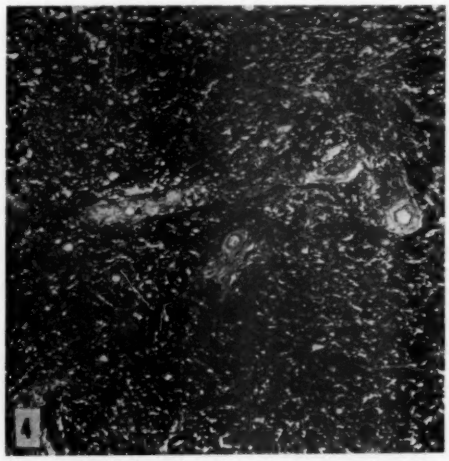


Fig. 4—Sagittal section through the neurohypophysis of a control dog. The aldehyde-fuchsin-positive material is evenly distributed in the tissue network. Gömöri's aldehyde-fuchsin stain; x 300.

places, the tissue network was almost devoid of granules (fig. 3). Material in dogs of this group also was clustered around the blood vessels. The scarcity of aldehyde-fuchsin-positive material in some areas made it difficult to determine the boundary between the posterior lobe and the rest of the hypophysis; in dogs in the control group, this boundary was distinct.

Discussion

The results showed that, in dogs with pyometra, the neurosecretory material decreased in amount in some parts of the hypothalamic-hypophysial system. As mentioned previously, the neurosecretory substance may contain the principles of the posterior lobe. The reduction of material probably can be explained only in two ways: The secretion of material, (*i.e.*, hormones in the nucleic ganglion cells) had decreased, or the release of hormones into the blood had increased to such an extent that the synthesis was not sufficient to compensate for it. The first possibility suggests a primary hypothalamic disturbance; the second could be due to a peripheral disturbance which causes an additional need of the posterior lobe hormones.

The clinical signs in dogs with pyometra indicate that there is a relatively small amount of the principles of the neurohypophysis in the organ. As early as 1947, Carlström [3] proposed the hypothesis that the low specific gravity of the urine was a result of the disturbances in the secretion of the hormones of the posterior lobe. Other clinical signs, primarily the affected tonus of the smooth musculature in the uterus, also suggest the lack of action of the principles of the posterior lobe.

It is impossible to determine what association these conditions have with other hormonal disturbances.

If water imbalance is related to hypothalamic disturbances, it seems obvious that the factors which cause these disturbances also produce other endocrine abnormalities. Further studies are in progress.

Summary

The hypothalamic-hypophysial systems of 6 bitches with pyometra and 10 healthy control bitches were studied, using Gömöri's aldehyde-fuchsin method. The amount of neurosecretory material had decreased in some parts of the neurosecretory system in bitches with pyometra. The connection of this condition with the eventual disturbance in the secretion of the principles of the posterior lobe was discussed.

References

[1] Baier, W., Kalich, J., and Taxacher, J.: Über Pathogenese und Behandlung der Endometritis bei Hündinnen. Berl. u. Münch. tierärztl. Wchnschr., *66*, (1953): 397.

[2] Bargmann, W.: Relationship Between Neurohypophysial Structure and Function. Proc. Colston Res. Soc., *8*, (1956): 11.

[3] Carlström, B.: Kompendium i medicin. Vet.-med. Fören. (Stockholm), *3*, (1947): 463–516.

[4] Dawson, A. B.: Evidence for the Termination of Neurosecretory Fibers Within the Pars Intermedia of the Hypophysis of the Frog, *Rana Pipiens*. Anat. Rec., *115*, (1953): 63.

[5] Gömöri, G.: Aldehyde-Fuchsin: A New Stain for Elastic Tissue. Am. J. Clin. Path., *20*, (1950): 665.

[6] Gömöri, G.: Observations with Differential Stains on Human Islets of Langerhans. Am. J. Path., *17*, (1941): 395.

[7] Halmi, N. S., and Davies, J.: Comparison of Aldehyde Fuchsin Staining, Metachromasia and Periodic Acid-Schiff Reactivity of Various Tissues. J. Histochem. & Cytochem., *1*, (1953): 447.

[8] Hetzel, H.: Die Hyperplasia glandularis cystica endometrii bei den Fleischfressern. Wien. tierärztl. Monatschr., *22*, (1935): 609.

[9] Hild, W.: Experimentelle-morphologische Untersuchungen über das Verhalten der "neurosekretorischen Bahn" nach Hypophysenstieldurchtrennungen, Fingriffen in den Wasserhaushalt und Belastung der Osmoregulation. Virchows Arch. f. path. Anat., *319*, (1951): 526.

[10] Kivalo, E., and Talanti, S.: The Neurosecretory Substance in the Hypothalamic-Hypophysial System of the Horse. Acta endocrinol., *26*, (1957): 128.

[11] Landing, B. H., Hall, H. E., and West, C. D.: Aldehyde-Fuchsin-Positive Material of the Posterior Pituitary. Lab. Invest., *5*, (1956): 256.

[12] Ortmann, R.: Morphologisch-experimentelle Untersuchungen über das diencephal-hypophysäre System im Verhältnis zum Wasserhaushalt. Klin.-therap. Wchnschr., *20*, (1950): 449.

[13] Scharrer, E., and Scharrer, B.: Die Neurosekretion, v. Möllendorffs Handbuch d. mikrosk. Anat. d. Menschen. (Heidelberg), *5*, (1954): 953.

[14] Schulze, W.: Zur Pymetra der Hündin. Deutsche tierärztl. Wchnschr., *62*, (1955): 504.

[15] Talanti, S.: Observations on the Hypothalamic-Hypophysial Neurosecretory System of the Cow. Nord. Vet.-med., *10*, (1958): 153.

[16] Teunissen, G. H. B.: The Development of Endometritis in the Dog and the Effect of Oestradiol and Progesterone on the Uterus. Acta endocrinol., *9*, (1953): 407.

XUM

The Use of Glycine-1-C^{14} in the Measurement of Serum Protein Turnover Rates in Dairy Cows

CHARLES E. CORNELIUS, D.V.M., Ph.D.; ARTHUR L. BLACK, Ph.D.; MAX KLEIBER, D.Sc.

Davis, California

METHODS employing the relative uptake of amino acids into tissue and serum proteins have been used in a multitude of investigations.[18, 27, 33, 34] Studies concerning methods used in the measurement of serum protein turnover rates in mammals have been made primarily on the rat,[7, 16] dog,[23, 41] and man.[11, 25]

Dixon *et al.*,[10, 11] using procedures employing transfused I^{131}-tagged albumin and *gamma* globulin, investigated serum protein turnover in steers and nonlactating cows. They found half-time ($t_{1/2}$) values for I^{131}-tagged albumin and *gamma* globulin in cattle to be 20.0 ± 1.1 and 21.2 ± 1.7 days, respectively.

Many investigators[4, 39] have obtained results, using C^{14}-labeled proteins, that conflict with values calculated using I^{131}-tagged serum proteins. In a study of iodine denaturation of plasma proteins, McFarlane[26] cast doubt on the validity of iodinated protein turnover data obtained with methods other than the "jet-iodination" technique. The coupling of large quantities of I^{131} may render the protein more or less subject to proteolytic attack. Denaturation of the serum proteins may, in addition, result from internal *gamma* irradiation from the I^{131}.[3]

The measurement of serum protein turnover has been performed by a variety of methods, each having specific advantages and disadvantages. A comprehensive review has been published by Tarver,[40] concerning problems encountered in the measurement of protein synthesis and turnover. Methods used previously in the measurement of serum protein turnover have been of three basic types.

In the first type, the labeled precursor compounds are fed or injected into animals and the disappearance of radioactivity in the serum proteins is followed over a period of days after maximum specific activity has been attained. This method has been referred to as the "direct" method[40] and is subject to error due to the continuous entry of label from the free amino acid pool into the serum proteins. In the second method,[20] animals are given radioactive amino acids in their feed, in an attempt to maintain a constant level of radioactivity in the precursor pool, and the ascending curve of the plasma protein specific activity is measured. In the third method, serum proteins are biologically labeled with radioactivity in one series of experimental animals and the labeled proteins transfused to the animal to be studied.[40] This method has been referred to as the "Hevesey" technique and was used extensively by Fink *et al.*[14]

This paper presents two methods in which the kinetics of serum protein turnover were studied by the injection of glycine-1-C^{14}. The serum protein replacement and turnover rates were estimated, using 3 cows to illustrate the utilization of such methods. The serum protein replacement and turnover rates were estimated by these methods for the intravascular serum protein compartment and the total "serum protein pool." Turnover rates for the intravascular serum protein compartment were calculated from a least squares regression line from the specific activities of the serum proteins between five and 35 days after the injection of glycine-1-C^{14}. Replacement rates of the total serum protein pool (intra- and extravascular) were calculated during the first 12 hours after injection by a procedure in which the radioactivity of the glycine in the hippuric acid is assumed to be equal to the radioactivity of the glycine entering the serum proteins.

MATERIALS AND METHODS

Glycine-1-C^{14} was injected through plastic tubing as described previously by Ralston *et al.*[30] Blood samples were taken from the cows hourly for the first three hours, then at 5, 10, 20, and 48 hours after injection. After this, blood samples were obtained weekly until 30 or more days had elapsed.

Urine samples were collected by perineal titillation or catheterization when voiding was incomplete. Total urine was collected for the first 48 hours, followed by random sampling every few days until the trial was complete. Urine samples

From the School of Veterinary Medicine and the Department of Animal Husbandry, University of California, Davis.

Prepared from a thesis submitted by the senior author to the University of California in partial fulfillment of the requirements for the Ph.D. degree.

This investigation presents results obtained by the Davis Tracer Team for Metabolic Research, supported in part by grants from the United States Atomic Energy Commission and the National Science Foundation.

The authors thank Jane Bishop and Martin Small for technical assistance during this investigation.

were stored at −10 C. until hippuric acid crystallization was performed.

Hippuric acid was crystallized from the urine by the method of Quick.[29] To insure that there was no contamination of the hippuric acid with free radioactive glycine in the urine, 15 μc. of glycine-1-C^{14} was added to a bovine urine sample which contained no radioactivity in the hippuric acid. Recrystallization two times from boiling water in an ice bath insured the absence of radioactivity from free urine glycine adsorbed on the hippuric acid. When urine samples had concentrations of hippuric acid below 100 mg./100 ml., the method of Griffith[17] was employed, using a continuous liquid ether extraction. The hippuric acid next was hydrolyzed by refluxing for eight hours in 6 N HCl, filtered through Whatman No. 40 filter paper and extracted three times with ether to remove the benzoic acid. The aqueous glycine was vacuum distilled to dryness, washing twice with distilled water. The glycine solution was decolorized, using charcoal (Norit) and filtered, and 1 ml. of propylene oxide was added upon cooling to allow for the conversion of the amino acid to its free base. The glycine was precipitated with ethanol[26] and washed with ether[18] as described previously.

The crystals first were air dried and then were transferred to a vacuum oven for drying at 45 C. The glycine was checked for purity on paper chromatograms, using H_2O-butanol-acetic acid (4:4:1) as the solvent system.[5, 26]

Serum proteins were prepared by ethanol precipitation[31] after the dialysis of 80 ml. of serum against distilled water, using Cenco cellophane tubing (2.5 mm. wide) for 48 hours with constant rotation at 1 r.p.m. in a 1-liter cylinder. By changing the distilled water ten times per 48 hours, all dialyzable nonprotein radioactivity was removed. The serum proteins were precipitated after replacing NaCl at 40 mg./10 ml. of serum by adding 95 per cent ethanol up to a concentration (V/V) of 50 per cent.[6] The precipitated serum proteins were washed twice with 150 ml. of warm alcohol-ether (3:1) to remove carotenoids and lipids, filtering each time through Whatman No. 1 paper. Finally, the proteins were mixed twice with anhydrous ether and dried at room temperature prior to complete drying in a vacuum oven. Methods used in the hydrolysis of serum proteins and the separation of the amino acids on Dowex-50 ion exchange columns were similar to those described previously by Stein and Moore.[28] All amino acids were checked for purity and identity on paper chromatograms, using either butanol-acetic acid-water or phenol-water solvent systems.[5]

All amino acids and proteins were combusted to CO_2 in a Sargent microcombustion apparatus and counted for radioactivity by methods described previously by Kleiber *et al.*[20, 21]

RESULTS AND DISCUSSION

Replacement and Turnover Rates of the Intravascular Serum Proteins.—The measurement of the turnover rate and $t_{1/2}$ values of the serum proteins following the injection of a labeled amino acid is based upon the assumption that the change in tracer concentration of the serum proteins is a "first order" process, *i.e.*, the logarithm of tracer concentration decreases as a linear function of time. In our study, this postulate involves the assumption that all labeled amino acids are lost in a random manner from a homogeneous system and that the loss of one labeled amino acid is representative of the loss of other amino acids within the protein molecule. During the first few days after injection, there is a rapid exchange of labeled serum proteins between the vascular, interstitial, and cellular compartments. After this exchange process no longer influenced the decrease in the specific activity of the serum proteins, the slope of the specific activity of the serum proteins was used to estimate the "metabolic" disappearance. Calculations in this study were made between five and 35 days after injection, at which time the slope of the logarithms of the specific activities of the total serum proteins plotted against time became nearly constant and suggested the biokinetics of a first order process.

By the method of least squares, a regression coefficient was calculated which represents the estimated turnover rate (k) of the serum proteins.[37]

The relationships between various kinetic parameters are shown:

$$(1) \qquad \frac{d\alpha}{dt} = k\alpha$$

$$(2) \qquad \frac{\ln 2}{t_{1/2}} = \frac{a}{A}$$

$$(3) \qquad T_t = \frac{1}{k} = \frac{A}{a}$$

$$(4) \qquad a = \frac{A}{T_t}$$

[k = turnover rate of serum proteins; α = specific activity of serum proteins; a = transfer (replacement) rate in grams of protein per unit of time; A = compartment size in grams of serum protein; and T_t = turnover time (average life span).]

In this study, the "serum pool" is defined to include both intra- and extravascular serum protein compartments. Since continuous exchange occurs between the

TABLE 1—Data from Experiments on Cows Injected Intravenously with Glycine-1-C^{14}

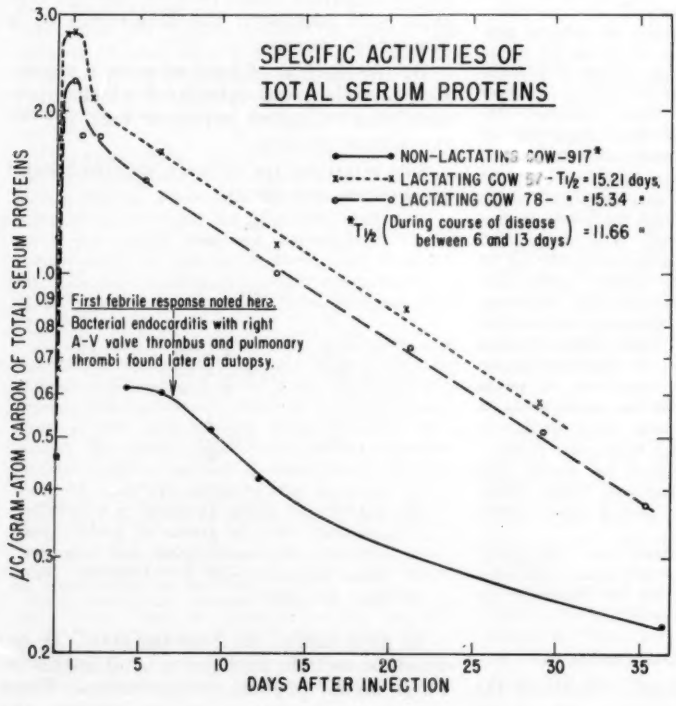
Cow (No.)	Age (years)	Weight (kg.)	Plasma volume (liters)	Injected dose (mc.)	Injected relative dose (μc./kg.)	Stage of lactation
917	Mature	383	16.9	1.50	3.92	Nonlactating
57	5.0	541	21.5	3.75	6.94	Lactating
78	4.5	449	22.5	4.26	9.48	Lactating

intra- and extravascular compartments, a turnover rate (formula 5) observed for the intravascular compartment should equal

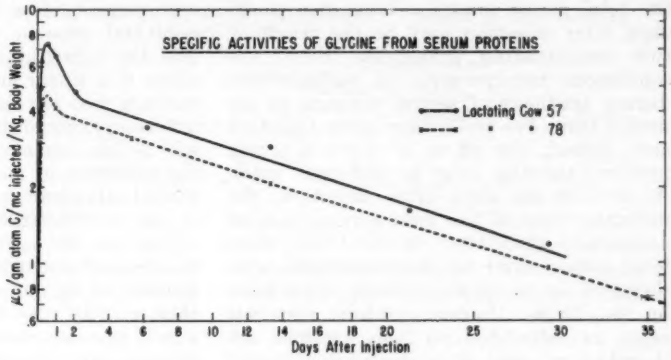
$$(5) \qquad \frac{da}{a} \Big/ dt$$

the turnover rate for the entire pool, after equilibrium of the tracer concentration between its compartments had been established ("A" and "a" refer only to the intravascular compartment). Compartment A was measured, using the dye T-1824, to compute the plasma volume, which then was multiplied by the serum protein concentration.

The specific activities of the total serum proteins and the serum protein glycine are presented (table 2; graphs 1, 2). Data concerning the turnover and replacement rates of serum proteins were calculated from the descending curves of the logarithms of the specific activities of both the total serum proteins and the serum protein glycine of the 2 lactating cows (table 3). Lactating cows 57 and 78, which were in similar stages of lactation following parturition, showed nearly identical turnover rates of 0.0455 and 0.0451 per day, respectively. However, their replacement rates (69.5 and 81.2 Gm./day) of intravascular serum proteins differed, since cow 78 contained a large intravascular compartment of serum proteins throughout the trial. The nonlactating cow, during the course of infection from multiple pulmonary thrombi, between six and 13 days after injection,



Graph 1—Specific activities of total serum proteins.


SPECIFIC ACTIVITIES OF GLYCINE FROM SERUM PROTEINS
Lactating Cow 57
78
μc/gm atom C/mc injected/Kg. Body Weight
Days After Injection

Graph 2—Specific activities of glycine from serum proteins.

showed a $t_{1/2}$ value for the total serum proteins of 11.66 days, with a turnover rate of 0.0593 per day. It was of special interest that the febrile nonlactating cow replaced intravascular serum proteins at a higher rate than lactating cow 57 and at nearly the same rate as lactating cow 78. The anorexia and inappetence associated with the disease probably provoked an increased protein catabolism which was manifested by a more rapid turnover rate of the serum proteins. Before the sixth day, the nonlactating cow appeared clinically normal and had a serum protein production rate of only 64 per cent that of the mean of the lactating cows, as measured by another method (table 6).

The half-time values of 15.21 and 15.34 days for total serum proteins in lactating cows 57 and 78 can only be compared with the limited data of Dixon *et al.*[10,11] of 20 ± 1.1 and 21 ± 1.1 days for albumin and *gamma* globulin in nonlactating cows and steers. Differences in the turnover rates of the individual serum proteins of cattle probably exist, as seen in other species. If the decreasing specific activity of plasma proteins in this study was not influenced by the continued incorporation of radioactivity as glycine or other amino acids into the serum proteins, then the experimental curve would represent the turnover rates of the serum proteins. The continued synthesis of serum proteins containing radioactivity from this free glycine pool must affect the slope of the curve of the specific activity of the serum proteins or serum protein glycine throughout the course of the investigations. Experimental evidence for this effect has been presented by Margen and Tarver,[24] using S^{35}-labeled cystine, in which they indicated that turnover rates of serum proteins are erroneously long if free S^{35}-labeled cystine is of significant concentration in the precursor amino acid pool.

The observed linearity of the curve of the logarithms of the specific activities of

TABLE 2—Total Serum Protein and Glycine of Serum Protein Standard Specific Activities

Nonlactating cow 917			Lactating cow 57				Lactating cow 78			
Time after injection		Standard specific activity* of total serum protein	Time after injection		Standard specific activity*		Time after injection		Standard specific activity*	
					Total serum protein	Glycine of serum protein			Total serum protein	Glycine of serum protein
Days	Hours		Days	Hours			Days	Hours		
0	2	0.151	0	2.5	0.0965	4.97	0	1	0.0486	1.14
4	7	0.157	0	5.0	0.259	6.33	0	2	0.123	2.39
6	7	0.155	0	11.5	0.398	7.31	0	3	0.154	3.16
9	7	0.132	1	0.0	0.403	6.59	0	5	0.201	4.28
12	7	0.107	2	1.0	0.329	4.80	1	0	0.239	4.14
36	7	0.047	6	7.0	0.242	3.46	1	12	0.192	.....
			13	7.0	0.164	2.84	2	13	0.191	.....
			21	7.0	0.124	1.62	5	7	0.156	3.18
			29	7.0	0.0858	1.19	13	7	0.107	2.03
							21	7	0.0772	.....
							29	7	0.0540	0.908
							35	7	0.0396	0.650

* μc./gaC/μc. injected/kg. body weight.

the total serum proteins from five to 35 days after injection may be the result of two compensating influences: First, the continuous incorporation of radioactivity during synthesis of serum proteins in the period from five to 35 days after injection and, second, the effect of various serum proteins turning over at different rates. At five or six days after injection, the precursor pool of free liver glycine is more radioactive than later in the trial. More label would enter in the continuous synthesis of serum proteins early than later in the trials. Serum protein turnover rates as calculated by this method are therefore minimal estimates of the "true" turnover rates of bovine serum proteins.

The degree of radioactivity in the free liver glycine pool, as compared with the glycine in the serum proteins, is presented (table 4). Five to six days after injection of the isotopic glycine, radioactivity of the free hepatic glycine pool, as measured from the urinary hippurate glycine, contained approximately one third the radioactivity present in the serum protein glycine. Four weeks after injection, both lactating cows still showed considerable radioactivity in the free glycine pool. Lactating cow 78 maintained a greater free glycine tracer concentration than was observed in lactating cow 57 in the later measurements of the trial (table 4).

The oversimplification of total plasma protein turnover has been criticized by Campbell *et al.*[7] They injected C^{14}-labeled and I^{131}-tagged plasma proteins into rats and found that the specific activity curve of total plasma proteins could not be expressed as a single exponential function at any stage. After injecting labeled albumin and *gamma* globulin, they[7] found that the logarithmic plot for each protein alone was linear but that the plot of the mixture was curved, since the sum of two different exponential functions can not itself be an exponential function. Without the influence of the continuous incorporation of label into the bovine serum proteins in our investigation, the logarithmic plots of the specific activities of the serum proteins might not have been linear. It would appear, in this study using glycine-1-C^{14}, that at least two interacting components, which are not themselves of a first order nature, may together produce a specific activity curve for the total serum proteins which simulates a first order process.

In comparing the turnover rates calculated from the specific activities of glycine from the serum proteins with that calculated from the specific activities of the total serum proteins, on the average a 13 per cent greater rate was observed when the glycine alone of serum proteins was the basis for the calculation. The turnover rate of glycine from serum proteins need not agree with the turnover rate of the total serum proteins. The two turnover rates should agree, however, if all the amino acids of the serum proteins had similar turnover rates and were affected similarly by the entrance of new label from the continuous synthesis of proteins. Since serine (55–65 per cent of total serum protein activity) and glycine (25–35 per cent of total serum protein activity) accounted for 80 to 90 per cent of the activity in the total serum proteins, a faster turnover rate for glycine than serine could account

TABLE 3—Intravascular Serum Protein Turnover Data (Calculated from the Specific Activities of Both the Total Serum Protein and Glycine of Serum Protein)

	Nonlactating cow 917	Lactating cow 57		Lactating cow 78	
Method of calculation	Serum proteins	Serum proteins	Glycine of serum proteins†	Serum proteins	Glycine of serum proteins†
$t_{1/2}$	11.66*	15.21	13.86	15.34	13.13
k (turnover rate/day)	0.0593	0.0455	0.050	0.451	0.0527
T_t (turnover time-days)	16.90	22.00	20.00	22.20	19.00
R (replacement or transfer rate) in Gm./day	80.20	69.50	76.40	81.20	95.30
Serum protein concentration (Gm./100 cc.)	8.00	7.10		8.00	
Serum volume (liters)	16.90	21.50		22.50	
Total intravascular serum protein (Gm.)	1,352.00	1,526.00		1,800.00	
Mean milk production (kg./day/kg. body weight)		3.20×10^{-2}		2.94×10^{-2}	

* Six to 13 days after injection during course of disease; † 100 Gm. of serum protein contains 2.98 Gm. of glycine.

TABLE 4—The Ratio of Radioactivity of Glycine from Serum Proteins to Glycine from Hippuric Acid

Lactating cow 57			Lactating cow 78		
Time after injection			Time after injection		
Days	Hours	Ratio*	Days	Hours	Ratio*
6	7	2.68/1	5	7	3.00/1
21	7	21.90/1	13	7	8.92/1
29	7	22.00/1	35	7	11.80/1

* $\frac{\mu c./gaC \text{ glycine from serum proteins}}{\mu c./gaC \text{ glycine from hippuric acid}}$

for the 13 per cent difference obtained. It also was observed that glycine accounted for a decreasing percentage of the total radioactivity in the serum proteins over a period of four weeks. As other amino acids of the serum proteins receive more radioactivity from metabolic interconversions, they would in time become a greater percentage of the total radioactivity present in the serum proteins.

Estimated Minimum Production Rates of the Total "Serum Protein Pool."—To estimate the minimum production rate of the total "serum protein pool" in this investigation, the minimum incorporation rate of glycine into the total (intra- and extravascular) serum protein pool was calculated during the first 12 hours after the injection of glycine-1-C^{14}. Since the liver appears to be involved in the synthesis of most of the serum proteins and of hippuric acid in most species, the specific activity of the glycine of the hippuric acid may equal that of the free glycine pool of the liver from which serum proteins are in part derived. There was assumed to be no significant delay in glycine conjugation to form hippuric acid and its subsequent excretion to the urine. A rapid conjugation and excretion time was substantiated by the injection of 30 Gm. of buffered sodium benzoate intravenously into nonlactating cow 97. An eightfold increase in the hippuric acid excretion rate was observed within the first 20 minutes after injection.

Snapper *et al.*[36] found only limited hippuric acid synthesis in perfused kidneys of ruminants, as compared with greater synthesis in the kidneys of human beings, pigs, and dogs. The studies of Bunge and Schmiedeberg,[6] followed by corroborating studies of Koche[22] and Bashford and Cramer,[2] have been interpreted as evidence that the kidneys of herbivores are of limited importance, as compared with the liver, in hippuric acid synthesis.

Henriques *et al.*,[18] investigating hippuric acid kinetics in the rabbit, presented data indicating that the specific activities of free liver glycine and of free serum glycine were nearly equilibrated in slightly less than one hour after its intravenous injection. The specific activity of the glycine obtained from the urinary hippuric

TABLE 5—Specific Activities of Glycine from the Urinary Hippuric Acid

Time after injection		Nonlactating cow 917	Time after injection		Lactating cow 57	Time after injection		Lactating cow 78
Days	Hours	(μc./gaC)	Days	Hours	(μc./gaC)	Days	Hours	(μc./gaC)
COMPOSITE SAMPLES								
....	0–1	2509.70	....	0–1	3328.10	....	0–1	3376.20
....	1–2	700.40	....	1–3	1366.70	....	1–2	3124.70
....	2–3	210.10	....	3–5	437.60	....	2–3	1226.70
....	3–5	130.70	....	5–11	188.30	....	3–5	585.70
....	5–10	68.92	....	11–24	61.96	....	5–10	261.70
....	10–20	50.00	....	24–49	22.59	....	10–24	99.45
....	20–30	41.83	....	49–59	19.91	....	36–49	30.03
....	30–40	14.54	....	.....	.....	....	.....	.....
....	40–60	8.82	....	.....	.....	....	.....	.....
RANDOM SAMPLES								
4	7	5.98	6	7	8.92	5	10	10.03
6	7	4.68	9	7	5.47	9	7	6.12
9	7	3.45	14	7	2.89	13	7	2.16
12	7	2.14	17	7	1.45	16	7	1.74
23	7	1.43	21	7	0.51	21	7	1.45
28	7	0.745	25	7	0.42	24	7	1.30
36	7	0.536	29	7	0.375	29	7	0.848
44	7	0.405	31	0	0.402	35	7	0.525
....	..	.....	....	..	.....	43	7	0.375

XUM

acid during specified time intervals was considered to represent the mean specific activity of the free liver glycine. Probably only one pool of free liver glycine exists in the liver for many synthetic processes. All radioactivity found in the serum proteins was interpreted as an index of net protein synthesis; however, the possibility of terminal molecular exchange of amino acids with no actual net protein synthesis can not be overlooked.

The presence of hippuric acid glycine in the urine originating from sites of synthesis other than the liver, and containing a lower or higher specific activity than the hippuric acid glycine derived from liver conjugation, could substantially affect the results in this method. Additional error may be present if the specific activity of the precursor glycine incorporated into *gamma* globulin in the reticuloendothelial tissue differs substantially from that found in the urinary hippuric acid.

An estimation of the magnitude of the total serum protein pool synthetic rate can be made if, in addition to the specific activity of the precursor glycine in a given time, the total amount of radioactivity incorporated into the serum proteins as glycine is known. Having determined the percentage of glycine in the serum proteins of these cows (2.98%), using Dowex-50 ion exchange chromatography,[5,38] a minimum total serum protein pool production rate may be estimated as indicated:

$$(6)\quad a = \frac{r}{p} = A\,\frac{(at_2 - at_1)}{\delta m \cdot p}$$

[t = time; a = serum protein production rate in grams in period t_1 to t_2; p = glycine concentration in serum proteins (Gm./100 Gm.); r = grams of glycine incorporated into the total serum protein pool in time t_1 to t_2; A = grams of glycine in serum protein vascular pool; α = specific activity of vascular serum protein glycine; and δm = mean specific activity of hippurate glycine in period t_1 to t_2.]

Formula 6 expresses the mathematical treatment of data in this method. Serum proteins of the total serum protein pool are produced mainly by the liver and introduced into the intravascular compartment, via the hepatic veins or thoracic duct, before their exchange with the extravascular spaces. If all the radioactive serum proteins produced by the liver, following the injection of glycine-1-C^{14}, remained in the intravascular compartment of the total serum protein pool, this method would estimate accurately the production rate of the total serum protein pool. The loss of serum proteins introduced into the intravascular compartment, however, has been estimated as nearly 14 per cent per hour in the lactating cow.[9]

The total serum protein pool production rate as expressed in formula 6 would, therefore, be a minimum value, since newly synthesized serum proteins containing label and introduced into the blood from the liver are lost steadily from the vascular pool, exchanging with extravascular serum protein containing little or no radioactivity. Since the maximum specific activity of the serum protein glycine was attained five to 12 hours after injection, calculations were based on samples taken before this.

The specific activities of the precursor hippurate glycine are presented (table 5). Additional data pertaining to the produc-

TABLE 6—Total Serum Protein Pool Production Rates Using Glycine from Hippuric Acid as the Precursor Tracer Concentration for Serum Protein Production

Cow	Intravascular compartment of serum protein glycine (Gm.)	Minimum glycine incorporated per hour into total serum protein pool (hr.)	(Gm.)	Mean minimum production rate of total serum protein pool (glycine = 2.98 Gm./100 cc. protein) (Gm./day)	Ratio of mean minimum production rate of total serum protein pool to replacement rate of intravascular serum protein compartment $\frac{\text{(Gm./day)}}{\text{(Gm./day)}}$
917					
Non-lac-tating	47.9	0.0–3.0	0.148	118.2	.....
		0.0–2.0	0.236		
57		2.5–5.0	0.326		
Lac-tating	45.5	5.0–12.0	0.254	218.0	3.16/1
		0.0–1.0	0.155		
78		1.0–2.0	0.210		
Lac-tating	54.0	2.0–3.0	0.317	235.0	2.90/1
		3.0–5.0	0.497		

tion rates of the total serum protein pool are presented (table 6). During a comparable three-hour period just after injection, nonlactating cow 917 produced 64 per cent of the average of the serum protein produced by the 2 lactating cows. Serum protein production of the total pool was expressed for a 24-hour period, assuming that the mean production rate estimated for a three-hour period would remain constant for the entire day. The differences in the calculated hourly rates of minimum protein production indicate either the influences of compartmental mixing or the changes in the rate of protein synthesis. If the apparent increase in rates of protein synthesis results from mixing phenomena, a similar rise in both lactating cows could be expected. A similar increase was observed in both lactating cows between two and one half and five hours, with no change after five hours in lactating cow 57. Wasserman *et al.*[43] observed, for dogs, that the thoracic duct returned 65 per cent of the protein that leaked from the capillaries and indicated that lymph protein was not simply plasma proteins but a fluid containing many tissue proteins.

The ratios of the estimated minimum serum protein of the total pool produced per day to the estimated replacement rate of the serum proteins of the intravascular compartment, as calculated from the decline in specific activity of the intravascular serum proteins, are presented (table 6). In both lactating cows, the ratio of the mean minimum production rates of the total serum protein pool by one method (table 6, column 4) to the replacement rates of the intravascular serum protein compartments by the previous method (table 3) was approximately 3:1. After sufficient time had elapsed for the specific activity of serum proteins throughout the total serum protein pool to reach a quasi-equilibrated state, the turnover rate in one compartment of the total serum protein pool would be valid for the total pool. Replacement rates of the serum proteins of individual compartments of the total pool would be proportional to their compartment sizes. If values presented by Hughs,[19] suggesting an avascular serum protein compartment three times the volume of the intravascular serum protein compartment, are valid for the lactating cow, a mean serum protein concentration of all avascular compartments, 65 per cent of their concentration in the intravascular compartment, would exist. According to Drinker,[12] the concentration of heart lymph in the dog amounts to nearly 60 per cent of the protein concentration in the serum.

SUMMARY

1) Two procedures using the intravenous injection of glycine-1-C^{14} in the measurement of serum protein replacement and turnover rates in cows are presented.

2) Serum protein replacement rates are measured both from the descending curve of the logarithm of the specific activities of the total serum proteins and by a procedure using the hippurate glycine as the precursor tracer concentration for serum protein production.

References

[1] Abdou, I. A., and Tarver, H.: Plasma Protein. I. Loss from Circulation and Catabolism to Carbon Dioxide. J. Biol. Chem., *190*, (1951): 769.

[2] Bashford, E., and Cramer, W.: Ueber die Synthese der Hippursaure in Thierkorper. Hoppe-Seylers Ztschr. physiol. Chem., *35*, (1902): 64.

[3] Berson, S. A., and Yalow, R. S.: Distribution and Metabolism of ^{131}I-Labeled Proteins in Man. Fed. Proc., *16*, (1957): 138.

[4] Berson, S. A., Yalow, R. A., Schreiber, S. S., and Post, J.: Tracer Experiments with ^{131}I-Labeled Human Serum Albumin: Distribution and Degradation Studies. J. Clin. Invest., *32*, (1953): 746.

[5] Black, A. L.: A Study of the Biosynthesis of Amino Acids in Dairy Cows from Carbonate and Fatty Acids Using Carbon-14 as a Tracer. Ph.D. thesis, University of California, Davis, 1951.

[6] Bunge, G., and Schmiedeberg, O.: Über die Bildung der Hippursäure. Arch. Exptl. Path. u. Pharmakol., *6*, (1876): 233.

[7] Campbell, R. M., Cuthbertson, D. P., Matthews, C. M., and McFarlane, A. S.: Behavior of ^{14}C and ^{131}I-Labeled Plasma Proteins in the Rat. Internat. J. Appl. Radiation, *1*, (1956): 66.

[8] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Molin, M., and Taylor, H. L.: Preparation and Properties of Serum Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. J. Am. Chem. Soc., *68*, (1946): 459.

[9] Cornelius, C. E.: Serum Protein Turnover Rates in Dairy Cows Using Carbon-14 Labeled Glycine. Ph.D. thesis, University of California, Davis, 1957.

[10] Dixon, F. J., Maurer, P. H., and Deichmiller, M. P.: Half-Lives of Homologous Serum Albumin in Several Species. Proc. Soc. Exptl. Biol. & Med., *83*, (1953): 287.

[11] Dixon, F. J., Talmadge, D. W., Maurer, P. H., and Deichmiller, M.: The Half-Life of Homologous *Gamma* Globulin (Antibody) in Several Species. J. Exptl. Med., *96*, (1952): 313.

[12] Drinker, C. K.: The Lymphatic System. Vol. 4, No. 2, Medical Sciences, Lane Medical Lectures, Stanford University Publications, University Series, Stanford University Press, Calif. (1942): 72.

[13] Fieser, F.: Experiments in Organic Chemistry. D. C. Heath and Co., San Francisco, Calif. (1941): 358.

[14] Fink, R. M., Enns, T., Kimball, C. P., Silberstein, H. E., Bale, W. F., Madden, S., and Whipple, G. H.: Plasma Protein Metabolism—Normal and Associated with Shock. J. Exptl. Med., *80*, (1944): 455.

[15] Forker, I. L., and Chaikoff, I. L.: Turnover of

Serum Proteins in Diabetes as Studied with ^{35}S-Labeled Proteins. J. Biol. Chem., *196*, (1952): 829.

[16] Friedberg, F., Tarver, H., and Greenberg, D. M.: The Distribution Pattern of Sulfur Labeled Methionine in the Protein and the Free Amino Acid Fraction of Tissues after Intravenous Administration. J. Biol. Chem., *173*, (1948): 355.

[17] Griffith, W. H.: Benzoylated Amino Acids in the Animal Organism. III. A Method for the Determination of Hippuric Acid and a Study of Hippuric Synthesis in Rabbits. J. Biol. Chem., *69*, (1926): 197.

[18] Henriques, O. B., Henriques, S. B., and Neuberger, A.: Quantitative Aspects of Glycine Metabolism in the Rabbit. Biochem. J., *60*, (1955): 409.

[19] Hughs, W. L.: Interstitial Proteins: The Proteins of Blood Plasma and Lymph. In "The Proteins." (Neurath, H., and Bailey, K., ed.) Academic Press, Inc., New York, N.Y. (1954): 663.

[20] Kleiber, M., and Edick, M.: A Respiration Apparatus for ^{14}C Studies with Cows. J. Anim. Sci., *11*, (1952): 61–71.

[21] Kleiber, M., Smith, A. H., and Black, A. L.: Carbonate as Precursor of Milk Constituents in the Intact Dairy Cow. J. Anim. Sci., *195*, (1952): 707.

[22] Koche, W.: Über eine Methode zur Bestimmung der Topographie des Chemismus im thierischen Körper. Arch. ges. Physiol., *20*, (1879): 64.

[23] Madden, R. E., and Gould, R. G.: The Turnover Rate of Fibrinogen in the Dog. J. Biol. Chem., *196*, (1952): 641.

[24] Margen, S., and Tarver, H.: On the Reutilization of Labeled Amino Acid from Body Protein in the Synthesis of New Plasma Protein. Pacific Coast Biochem. Conf., Univ. of California, Berkeley, 1957.

[25] Masouredis, S. P., and Beckmans, M. L.: Comparative Behavior of ^{131}I and ^{14}C-Labeled Albumin in Plasma of Man. Proc. Soc. Exptl. Biol. & Med., *89*, (1955): 398.

[26] McFarlane, A. S.: Labeling of Plasma Proteins with Radioactive Iodine. Biochem. J., *62*, (1956): 135.

[27] Miller, L. L., Bale, W. F., Yuile, C. L., Master, R. E., Tishkoff, G. J., and Whipple, G. H.: The Use of Radioactive Lysine in Studies of Protein Metabolism. Biochem. J., *90*, (1949): 297.

[28] Niklas, A., and Maurer, W.: Über die Neubildung einzelner getrennter Serum-Erweich.-Fraktioner nach oraler Gabe von ^{35}S-L-Methionin an Ratten. Biochem. Ztschr., *323*, (1952): 89.

[29] Quick, A. J.: Clinical Application of Hippuric Acid and Prothrombin Test. Am. J. Clin. Path., *10*, (1940): 222.

[30] Ralston, N. P., Kleiber, M., and Smith, A. H.: Venous Catheterization of Dairy Cows. J. Dai. Sci., *32*, (1949): 889.

[31] Rittenberg, D.: The Application of the Isotope Technique to the Study of the Metabolism of Glycine. Cold Spring Harbor Symp. Quant. Biol., *13*, (1948): 173.

[32] Rittenberg, D., Schoenheimer, R., and Keston, A. S.: Studies in Protein Metabolism. IX. The Utilization of Ammonia by Normal Rats on a Stock Diet. Cold Spring Harbor Symp. Quant. Biol., *128*, (1939): 603.

[33] Schoenheimer, R., Ratner, S., and Rittenberg, D.: Studies in Protein Metabolism. VII. The Metabolism of Tyrosine. J. Biol. Chem., *127*, (1939): 333.

[34] Schoenheimer, R., Ratner, S., and Rittenberg, D.: Studies in Protein Metabolism. X. The Metabolic Activity of Body Proteins Investigated with L-Leucine Containing Two Isotopes. J. Biol. Chem., *130*, (1939): 703.

[35] Shemin, D.: The Biological Conversion of L-Serine to Glycine. J. Biol. Chem., *162*, (1946): 297.

[36] Snapper, I., Grunbaum, A., and Neuberg, J.: Über die Hippursäresynthese in der überlebenden Niere von verschiedenen Tiergattungen, und vom Menschen. Biochem. Ztschr., *145*, (1924): 40.

[37] Solomon, A. K.: Equations for Tracer Experiments. J. Clin. Invest., *28*, (1949): 1927.

[38] Stein, W. H., and Moore, S.: Chromatographic Determination of Amino Acid Composition of Proteins. Cold Spring Harbor Symp. Quant. Biol., *14*, (1949): 179.

[39] Sterling, K.: Serum Albumin Turnover in Laennec's Cirrhosis as Measured by ^{131}I-Tagged Albumin. J. Clin. Invest., *30*, (1951): 1238.

[40] Tarver, H.: Peptide and Protein Synthesis, Protein Turnover. In "The Proteins." (Neurath, H., and Bailey, K., ed.) Vol. 2. Academic Press, Inc., New York, N.Y. (1954): 1199.

[41] Tarver, H.: Radioactive Sulfur and Its Application in Biology. Advances Biol. & M. Phys., *2*, (1951): 281.

[42] Tarver, H., and Morse, L.: The Release of the Sulfur from the Tissues of Rats Fed Labeled Methionine. Advances Biol. & M. Phys., *173*, (1948): 53.

[43] Wasserman, K., and Meyerson, H. S.: Exchange of Albumin Between Plasma and Lymph. Am. J. Physiol., *165*, (1951): 15.

A Comparative Study of Lipid Accumulation in the Adrenal Glands of Mature Nonpregnant Dairy Heifers, Nonpregnant Lactating Dairy Cows, and Pregnant Lactating Dairy Cows

J. THOMAS BELL, Jr., D.V.M., Ph.D., and ALVIN F. WEBER, D.V.M., Ph.D.

St. Paul, Minnesota

IT IS ESTABLISHED that the adrenal glands of common laboratory animals, such as the rat, rabbit, and guinea pig, are lipid rich, *i.e.*, the cortices contain large amounts of sudanophilic material.[16, 22, 32] However, in cattle, sheep, swine, and the golden hamster, there is little or no sudanophilic material demonstrable.[1, 16, 28] The reason for the lipid-poor condition of the adrenal cortex in this group of animals is not known, but it has been suggested by Verne and Herbert [28] that the adrenal hormones of these animals are secreted immediately on production and that lipid materials are not stored either as precursors or as active hormonal substances.

On occasion, it is seen that adrenal glands from apparently normal cows have an increase in cortical lipids. This lipid material may either be distributed throughout the entire cortex or be confined largely to one of the zones. In metabolic diseases, such as ketosis and parturient paresis in cows and pregnancy toxemia of sheep, the adrenal cortex becomes enlarged and markedly lipid rich. Shaw *et al.*[25] and Groenewald *et al.*[7] described this as a fatty infiltration with partial degeneration of the cortical zones. Although all zones of the cortex may be filled with lipid material, the zona glomerulosa generally shows the greatest involvement.

Ketosis in cattle is primarily a postparturient disease, but lactation and pregnancy have been reported to be predisposing factors.[6, 9, 15, 21] Groenewald *et al.*,[7] in a discussion of the pathology of "domsiekte" (pregnancy disease) in sheep, suggested that the clinical manifestations of this disease probably result from a summation of the metabolic and morphological changes which exceed the tolerance level of the animal involved. If this theory can be applied to the cow, lactating and late pregnant cows would be most likely to show adrenal changes toward the ketotic type.

There are many discussions available on bovine ketosis and, also, brief descriptions of the adrenal glands of ketotic cows. There is no work, however, which deals directly with the kind and degree of cytological and morphological changes found in the adrenal glands of normal lactating and late pregnant cows.

The studies to be reported here were undertaken to test the hypothesis that the adrenal glands of lactating and pregnant cows undergo definite cytological and morphological changes similar to those seen in the adrenal glands of ketotic cows.

MATERIALS AND METHODS

The adrenal glands from 94 Holstein-Friesian cows and mature heifers were used in this study. The cattle were classified into three groups: (1) nonpregnant, nonlactating, mature heifers; (2) nonpregnant, lactating cows; and (3) late pregnant, lactating cows (table 1). The following is pertinent information concerning each group:

Group 1.—Adrenal glands were collected from 34 nonpregnant, nonlactating, mature heifers. The mammary glands from these animals were small and nonfunctional as in nulliparous animals and, on uterine examination, there were no signs of pregnancy. Therefore, the adrenal glands were free from the physiological stresses of lactation and pregnancy and constituted the control group.

Group 2.—These glands were collected from 31 nonpregnant, lactating cows with large udders which were grossly free from mastitis and which showed moderate to large quantities of milk when the parenchyma was cut. The uterus from each cow was examined to be sure that the animal was not pregnant.

Group 3.—The adrenal glands were collected from 29 late pregnant, lactating cows. The fetus from each cow measured at least 75 cm. (forehead-rump measurement).[23] The criteria for the mammary glands of the animals in this group were the same as for group 2.

All collections were made during the months from November to March, to eliminate any possibility of seasonal variation. The glands were

From the Division of Veterinary Anatomy, College of Veterinary Medicine, University of Minnesota, St. Paul. Approved for publication as scientific journal series article 3888, from the Minnesota Agricultural Experiment Station.

Dr. Bell is now with the Department of Veterinary Anatomy, Iowa State College, Ames.

Taken from a thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree.

The authors acknowledge the technical assistance of Miss Peggy McConnell, technician, and Miss Mary Phillips, junior scientist, of the Division of Veterinary Anatomy; and also thank Swift and Co., and the Superior Packing Plant, St. Paul, Minn., for their cooperation in the collection of the adrenal glands used in this study.

TABLE 1—Design of Experiment and Average Adrenal Gland Weights

Groups	No. animals	Animal weight range (lb.)	Average single adrenal weight (Gm.)
1—Nonpregnant, nonlactating heifers	34	800–1,100	9.1 ± 0.6
2—Nonpregnant, lactating cows	31	1,100–1,400	16.6 ± 0.8
3—Late pregnant, lactating cows*	29	1,100–1,400	14.6 ± 0.8

* Past seventh month of pregnancy.
± s.e. = $8/\sqrt{N}$.

collected from local slaughterhouses at the time of evisceration. All animals had passed antemortem and postmortem federal inspections and, on this basis, were assumed to be healthy. Thus, if adrenal gland differences were observed, the stress of an existing clinical disease process could be eliminated as a causative agent.

No carcass weights were available. However, the animals in groups 2 and 3 were estimated to be of comparable average weight (1,100-1,400 lb.), and those in group 1 were estimated to be about 300 lb. lighter.

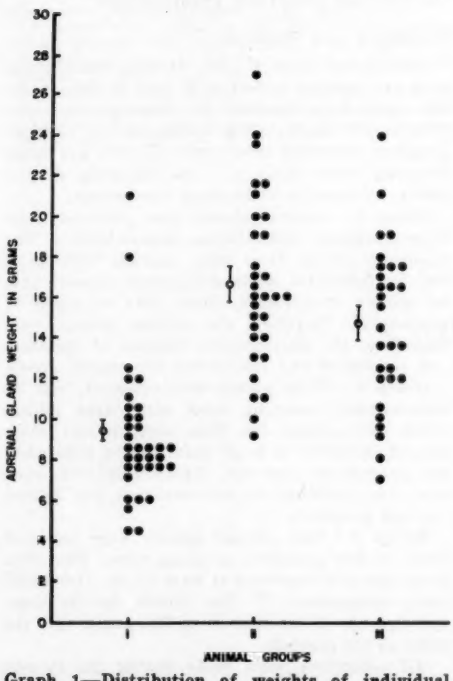
Tissue Preparation Techniques.—The adrenal glands were trimmed of all excess body fat and weighed on a triple-scale beam balance to the nearest 0.5 Gm. Average single adrenal gland weights were calculated for each group and an analysis of variance[27] was used to test for significance of differences.

Cross sections from the midportion of each gland were taken within a few minutes of collection. Tissues to be used for cytological studies were placed in mercury-formol-saline solution,[4] while those for studies of fat, birefringence, and qualitative cholesterol were placed in calcium-cadmium-formol.[2] All paraffin sections were cut at 7 μ and stained with Crossman's modification of Mallory's connective tissue stain.[3]

Studies made on the relative cortical cell sizes and on the absolute and relative thicknesses of the zona glomerulosa and zona fasciculata were done according to the method used by Weber *et al.*[23]

Oil red O, prepared in an aqueous medium (technique to be reported later), was used on fixed-frozen sections for the demonstration of intracellular lipid material. A modified Schultz test[21] was used for qualitative determination of cholesterol.

The presence of acicular birefringent particles in the cortices was determined by examination of fixed-frozen sections with a polarizing microscope.*



Graph 1—Distribution of weights of individual adrenal glands from mature nonpregnant heifers (group 1), nonpregnant lactating cows (group 2), and pregnant lactating cows (group 3).

RESULTS

Adrenal Gland Weights.—The average single adrenal gland weight for the nonpregnant, nonlacting heifers (group 1) was 9.1 ± 0.6 Gm.; for the nonpregnant, lactating cows (group 2), 16.6 ± 0.8 Gm.; and for the late pregnant, lactating cows (group 3), 14.6 ± 0.8 Gm. (table 1). The distribution of the individual adrenal gland weights from all three groups is shown (graph 1). It can be seen that the adrenal gland weights of the lighter animals in group 1 were concentrated within a narrow range (4.5–12.5 Gm.), while those of the heavier animals from groups 2 and 3 showed a wider distribution (7.0–27.0 Gm.). Analysis of variance showed significant differences in the adrenal gland weights between all groups studied. (P values between groups were as follows: 1 vs. 2 and 1 vs. 3, $P = <0.01$; 2 vs. 3, $P = <0.05$.)

Widths of Cortical Zones.—The average width of the zona glomerulosa in the adrenal glands of the 34 heifers in group 1 was 241 ± 10 μ (table 2), while the average width of this zone in the nonpregnant lactating cows (group 2) was 315 ± 12 μ. This difference is highly significant—241 ± 10 versus 315 ± 11 ($P = <0.01$).

The zona glomerulosa thickness in the

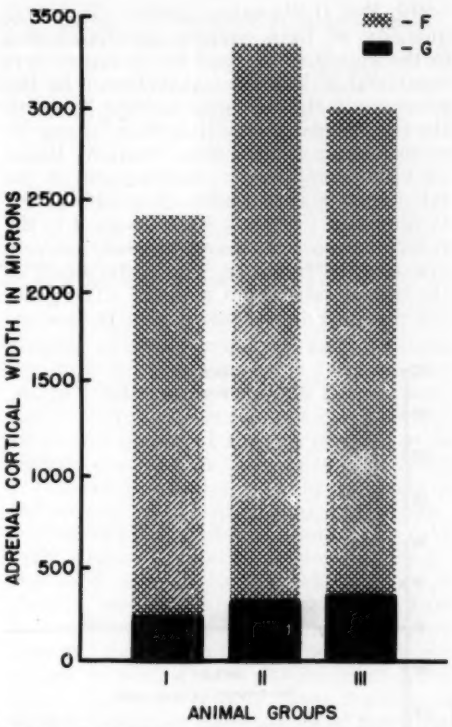
* Model No. P42AD, American Optical Co., Buffalo, N.Y.

late pregnant, lactating cows (group 3) was 346 ± 20 μ, and this increase, like that found in the cows in group 2, is highly significant when compared with that in cows of group 1. The difference in the average thickness of the zonae glomerulosae of cows in groups 2 and 3 is not statistically significant. The relative differences in zonae glomerulosae thicknesses for all three groups are shown (graph 2).

The average thicknesses of the zonae fasciculatae were 2,098 ± 67 μ for group 1, 3,012 ± 83 for group 2, and 2,640 ± 92 for group 3 (table 2). The difference between groups 1 and 2 was statistically significant, as was that between groups 1 and 3 (P = <0.01). There was no significant difference in the zonae fasciculatae widths in cows of groups 2 and 3. The relative contributions of the zonae glomerulosae and fasciculatae to the total cortical width in the adrenal glands of all three groups of animals are shown (graph 2).

On calculating the ratios of zona fasciculata widths to zona glomerulosa widths (table 2), it was found that the heifer group ratio (8.7) occupied a position which was intermediate between that of the lactating (9.6) and the pregnant lactating cows (7.6). All these ratios were subjected to statistical analysis and only the ratio differences between the pregnant lactating and the nonpregnant lactating cows were highly significant (P = <0.01).

Relative Sizes of Parenchymal Cells in the Cortical Zones.—Counts were made of the number of cell nuclei per unit area (784 sq. μ) in the cortical zones of all adrenal glands studied, to determine whether a change occurred in relative sizes of zonae glomerulosae and fasciculatae cells with an increase in the weight of the glands (table 2). In the cows in group 1, the average nuclei values were 6.4 ± 0.08 in the zona glomerulosa and 3.8 ± 0.07 in the zona fasciculata. In those in group 2, they were 5.9± 0.18 and 3.1 ± 0.07; in those in group 3, they were 6.0 ± 0.18 and 3.6 ± 0.09. The differences in average numbers of nuclei in comparable zones of cows in the three groups studied were not statistically significant.



Graph 2—Relative contributions of the zonae glomerulosae (G) and zonae fasciculatae (F) (includes zonae reticulares) to the total cortical width in the adrenal glands from mature nonpregnant heifers (group 1), nonpregnant lactating cows (group 2), and pregnant lactating cows (group 3).

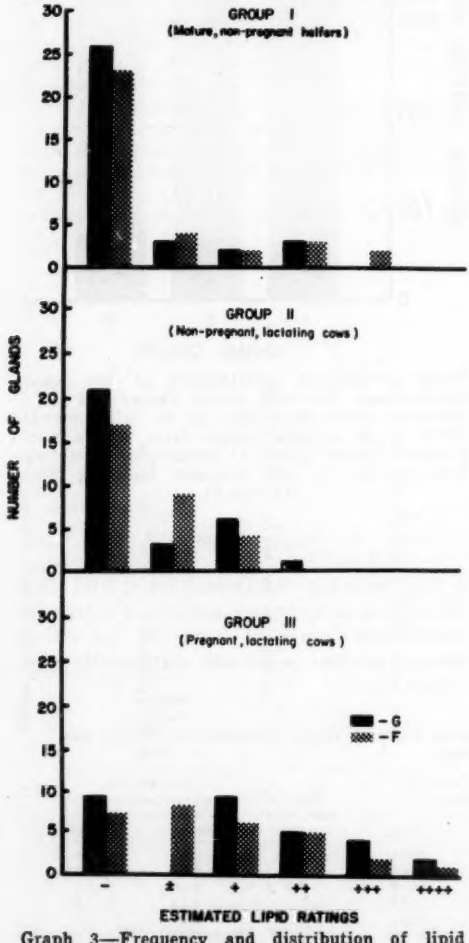
TABLE 2—Average Widths and Ratios of Adrenal Cortical Zones (Numbers of Nuclei per Unit Area)

Groups	No. glands	Average width of cortical zones (μ)		Ratio of fasc./glom. widths	Average No. nuclei/unit area*	
		Glom.	Fasc.		Glom.	Fasc.
1—Nonpregnant, nonlactating heifers	34	241 ± 10	2098 ± 67	8.7	6.4 ± 0.08	3.8 ± 0.07
2—Nonpregnant, lactating cows	31	315 ± 11	3012 ± 83	9.6	5.9 ± 0.18	3.1 ± 0.07
3—Late pregnant, lactating cows	29	346 ± 20	2640 ± 92	7.6	6.0 ± 0.18	3.6 ± 0.09

* 784 sq. μ.

± s.e. = $S/\sqrt{N}$; glom. = zona glomerulosa; fasc. = zona fasciculata.

Oil Red O-Stainable Lipids.—The total quantity of lipid droplet material found in the glands of the heifers (group 1) was considerably less than that found in the pregnant lactating cows (group 3), with the nonpregnant, lactating cows (group 2) occupying an intermediate position. Based on visual microscopic examinations of the fat-stained glands, values of negative (−) to four plus (++++) were assigned to the individual cortical zones, to quantitatively evaluate the frequency and distribution of the accumulated lipid material. The gradual change in lipid content from the mature heifers (group 1) to the pregnant, lactating cows (group 3) is shown (graph 3).



Graph 3—Frequency and distribution of lipid ratings in the zonae glomerulosae (G) and zonae fasciculatae (F) of adrenal glands of cattle in groups 1, 2, and 3.

It was found that, as the total quantity of adrenal lipid droplet material increased from the mature heifers to the pregnant lactating cows, the zonae glomerulosae showed a slightly greater tendency toward lipid accumulation than did the zonae fasciculatae (table 3). In glands which showed accumulations of lipid droplet material in both zones of the cortex, those droplets in the zona glomerulosa were generally of a larger size (up to 5 μ) than those in the zona fasciculata. The droplets in the zona fasciculata were more constant in size, generally measuring up to 3 μ although, in some glands, lipid droplets were comparable in size to those seen in the zona glomerulosa. The reason for the difference in size of the lipid droplets of the zonae glomerulosae and fasciculatae is not known.

Occurrence, Amount, and Distribution of Acicular Birefringent Crystals.—Long acicular, or needle-shaped, birefringent crystals were seen in the cortices of the adrenal glands in all three groups of cattle studied. The crystals varied from 2 to 3 μ to 25 μ in length, and from less than 1 to 2 μ in width. These particles could be found throughout the zonae glomerulosae and fasciculatae, but tended to be more numerous in the inner portion of the zona glomerulosa and the outer portion of the zona fasciculata (fig. 1). However, they were found consistently in greater quantities in the zona glomerulosa than in the zona fasciculata (table 3). Birefringent particles were found in the zona glomerulosa of every adrenal gland examined while, in 30 of the total of 94 glands studied, no acicular crystals were seen in the zona fasciculata.

The presence and location of the needle-shaped, birefringent crystals in this study were correlated only roughly with the presence and location of oil red O-stainable material and could not be related to the presence of cholesterol.

Qualitative Adrenal Gland Cholesterol.—A modification of the Schultz histochemical test for cholesterol [31] was carried out on frozen sections from all adrenal glands. In every adrenal gland studied in all three groups, the cortex was negative to the test (table 3). The myelinated nerves, with which the medulla of the bovine adrenal gland is well supplied, always were positive and served as a control for the reaction.

TABLE 3—Estimations of the Average Quantitative Ratings for Oil Red O-Stainable Lipid, Birefringent Crystals, and Cholesterol Reaction in the Adrenal Cortex

Groups	No. glands	Oil red O*		Birefringence		Cholesterol† reaction	
		Glom.	Fasc.	Glom.	Fasc.	Glom.	Fasc.
Mature, nonpregnant heifers (group 1)	34	−	−	++	+	−	−
Nonpregnant, lactating cows (group 2)	31	+	±	+++	+	−	−
Pregnant, lactating cows (group 3)	29	++	+	+++	++	−	−

* The plus and minus signs are based on visual microscopic inspection and are to be interpreted as follows: − indicates the absence of stainable lipid material; +++ indicates that the cells contained considerable quantity of droplet material. † Positive reaction in the medullary nerves served as control. Glom. = zona glomerulosa; fasc. = zona fasciculata.

Discussion

Part of the average weight increases of the adrenal glands of cows in groups 2 and 3 over those of adrenal glands of cows in group 1 probably is due to normal growth of the gland,[13] since these were from older and heavier animals. However, it is unlikely that normal growth alone would account for the large differences found in this study. The stresses of lactation and pregnancy are, apparently, the major contributing factors to the increase in weight of the adrenal glands of cows in groups 2 and 3. Randall and Graubard [18] and Hewitt and Van Liere [10] have demonstrated that the adrenal glands of rats under the stresses of lactation and pregnancy show increases in weight over the adrenal glands of nonpregnant, nonlactating rats, and Krupski [13] found that the hypertrophy seen in adrenal glands of multiparous cows was due to the effects of lactation. However, Krupski's observations were based on gross examination, rather than on actual weights with statistical analysis.

It was expected that adrenal glands of cows in group 3, which were under the stresses of both pregnancy and lactation, would be heavier than those from cows in group 2, which were lactating but nonpregnant. However, this was not the case. Instead, the glands of cows in group 3 were slightly smaller than those in cows in group 2. It is possible that, due to the acuteness of the stresses of late pregnancy, parturition, and lactation, the adrenal gland during the early stages of lactation becomes markedly increased in size over that which occurred during pregnancy but, as lactation proceeds and its stressor effect becomes more chronic, the adrenal gland gradually decreases in size, due to physiological adaptation. Since the cows in group 2 probably were in relatively early stages of lactation, their adrenal glands might still be showing slight effects of the original physiological hypertrophy.

The possible effects of fetal adrenal hormonal augmentation on the size and function of the maternal adrenal gland must be considered. The pituitary-adrenal system is well developed and functional at the seventh month of fetal development. It is possible, therefore, that fetal cortical hormones, under stimulation of the pituitary

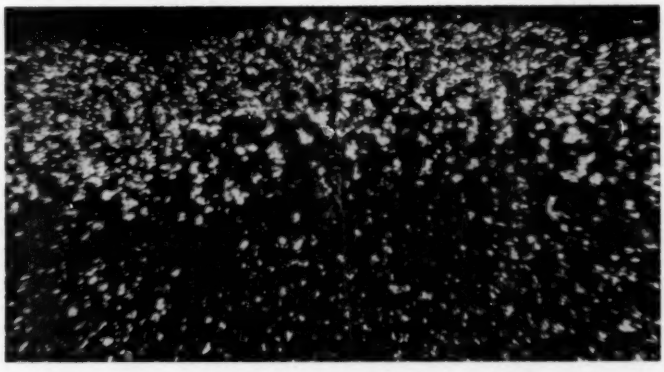


Fig. 1—Section of the adrenal gland from a late pregnant, lactating cow, showing a marked accumulation of birefringent crystals in the zona glomerulosa (above). The zona fasciculata (below) shows a lesser amount. x 100.

XUM

gland, could supplement those of the mother and thus modify the maternal stimulus for an increase in adrenal gland size. Although there is no direct evidence to support this theory, there is evidence of a fetal adrenal gland response to a maternal adrenal gland insufficiency.[11, 12, 29]

The significant downward shift of the ratios of the zona fasciculata:zona glomerulosa widths from 9.6 in the nonpregnant lactating cow to 7.6 in the late pregnant lactating cow suggests that the lack of proportional increase in size of the zona fasciculata as compared with that of zona glomerulosa in adrenal glands of pregnant lactating cows probably was responsible for the decrease in the average adrenal gland weight. This peculiar behavior on the part of the zona fasciculata in the glands of the group 3 animals is difficult to explain. It could be related to physiological adaptation, to a decrease in the amount of stress inherent in lactation, to the influence of either the fetal adrenal corticoids or the placental hormones on gonadal hormone augmentation, or to all of these. More work needs to be done on fetal-maternal relationships before this question can be answered.

In all three groups studied, the proportion of zona glomerulosa to zona fasciculata cell nuclei remained about two to one (table 2). This means that, under the conditions of this study, with the increase in size of the adrenal glands of cows in groups 2 and 3, there was no increase in zonal cell size over that of the lighter adrenal glands in the mature heifers (group 1). Although no mitosis was observed in the sections studied, it was presumed that the increase in size of the adrenal cortical zones was due to a slow rate of cellular hyperplasia (*i.e.*, increase in cell numbers) rather than to cellular hypertrophy (*i.e.*, increase in cell size).

If cell size is considered a reliable criterion of the degree of cellular activity in the adrenal cortex,[5] then one should expect to find larger cortical cells in animals under the stresses of lactation and pregnancy than in heifers. However, no such differences were found.

Results of the oil red O lipid study indicate that, with pregnancy and the further stress of lactation, there is a gradual accumulation of lipid material within the cells of the cortical zones. It was observed further that the zona glomerulosa had a greater tendency toward lipid accumulation than did the zona fasciculata. In cases of clinical ketosis, the zona glomerulosa also shows greater accumulations of lipid material and more pathological changes than the zona fasciculata.[25, 26] If the degree of lipid accumulation observed in the adrenal cortices of the pregnant lactating cows was increased three- or fourfold, it would parallel the condition which is characteristic of bovine and ovine ketosis. This lipid accumulation is believed to represent evidence of cellular inactivation, since lipid accumulation was produced in experiments specifically designed to inactivate the zona glomerulosa of the calf adrenal gland.[30] However, because of the normally lipid-poor characteristic of the bovine adrenal cortex, it is difficult to draw any conclusions as to its functional state based on findings which parallel those described for the rat,[5] where the gland is normally lipid rich.

At the present time, no interpretations can be made as to the nature or significance of the acicular birefringent crystals. They were correlated only roughly with the presence of cholesterol. These particles can be demonstrated only in fixed-frozen material and are considered extracellular artifacts [30] which probably result from crystallization of endogenous lipid material of unknown chemical nature.

A negative Schultz cytochemical test for the qualitative determination of 7-OH cholesterol is a constant feature of the normal bovine adrenal cortex. This also was true for all the glands in this study, including those in which there was considerable stainable lipid material present. The negative test does not indicate that there is no cholesterol present, but that the concentration of its oxy- derivative is less than 12 to 25 per cent in the lipid phase.[19]

Schultz-positive material is demonstrated easily in the adrenal cortices of animals such as the rabbit and guinea pig, and the increase and decrease in the intensity of the reaction has been used as a tool to correlate the rise and fall of cholesterol content with adrenal activity.[17, 32] This correlation is impossible to study in the cow at present, due to the normally consistently negative reaction of the bovine adrenal gland to the available cytochemical tests for cholesterol.

SUMMARY AND CONCLUSIONS

The adrenal glands from 34 mature nonpregnant dairy heifers, 31 nonpregnant

lactating dairy cows, and 29 late pregnant lactating dairy cows were studied in an effort to learn more about the incidence and nature of lipid accumulation in the bovine adrenal gland under the stresses of pregnancy and lactation.

The average adrenal gland weights of nonpregnant lactating (16.6 ± 0.8 Gm.) and pregnant lactating (14.6 ± 0.8 Gm.) cows were significantly greater ($P = <0.01$) than the average weights of the mature, nonpregnant heifers (9.1 ± 0.6 Gm.).

The average adrenal gland weight of the nonpregnant lactating cow was significantly greater ($P = <0.05$) than that of the pregnant lactating cow. The smaller adrenal glands in the pregnant lactating cows might be the result of physiological adaptation to the stresses of pregnancy and lactation, or to adrenal supplementation by progesterone, or placental or gonadal hormones, or to fetal adrenal hormone production.

The widths of the zonae glomerulosae and fasciculatae in the adrenal glands of the nonpregnant lactating and pregnant lactating cows were significantly greater ($P = <0.01$) than those of the mature nonpregnant heifer.

The widths of the zonae glomerulosae in adrenal glands of the nonpregnant lactating (group 2) and pregnant lactating (group 3) cows were not significantly different. Conversely, the widths of the zonae fasciculatae of the glands of cows in group 2 were significantly greater than those for group 3 cows ($P = <0.01$). The lack of proportional increase in the size of the zona fasciculata as compared with the zona glomerulosa in the adrenal glands of cows in group 3 could have been responsible for the decrease in average adrenal gland weight.

There were no significant differences in the numbers of nuclei per unit of cortical zone area in either the zona glomerulosa or zona fasciculata among the three groups of animals studied. Under the conditions of this study, it is believed that the increase in the size of the adrenal glands of cows in groups 2 and 3 was due to cellular hyperplasia rather than to cellular hypertrophy.

No lipid-laden adrenal glands characteristic of those described in studies of bovine ketosis were found in any of the animals. Amounts of oil red O-stainable lipid material in the glands gradually increased from group 1 cows to group 3 cows, with the zona glomerulosa showing a greater tendency toward lipid accumulation than the zona fasciculata.

Acicular birefringent particles were found in all adrenal glands studied, with the zonae glomerulosae showing more involvement than the zonae fasciculatae. There appeared to be no correlation between quantity of birefringent particles and quantity of oil red O-stainable material.

All glands studied were negative to the Schultz cytochemical test for cholesterol in both the zona glomerulosa and zona fasciculata.

On the basis of this study, it appears that the stresses of pregnancy and lactation bring about a gradual increase in the lipid content of the bovine adrenal gland. Although the lipid content of the glands from the pregnant lactating cows appeared to be much less than that seen in cows with ketosis and other parturient diseases, the pattern of distribution of the lipid material resembled that seen in cows with these postparturient conditions.

References

[1] Alpert, M.: Observations on the Histopathology of the Adrenal Gland of the Golden Hamster. Endocrinology, *46*, (1950): 166–176.

[2] Baker, J. R.: The Structure and Chemical Composition of the Golgi Element. Quart J. Micros. Sci., *85*, (1944): 1–72.

[3] Crossman, G.: A Modification of Mallory's Connective Tissue Stain with a Discussion of Principles Involved. Anat. Rec., *69*, (1937): 33–38.

[4] Dawson, A. B., and Friedgood, H. B.: Differentiation of Two Classes of Acidophiles in the Anterior Pituitary of the Female Rabbit and Cat. Stain Tech., *13*, (1938): 17–21.

[5] Deane, Helen W., Shaw, J. H., and Greep, R. O.: The Effects of Altered Sodium or Potassium Intake on the Width and Cytochemistry of the Zona Glomerulosa of the Rat Adrenal Cortex. Endocrinology, *43*, (1948): 133–153.

[6] Forbes, R. M.: Ketosis in Ruminants. Cornell Vet., *33*, (1943): 27–47.

[7] Groenewald, J. W., Grof, H., Bekker, P. M., Meian, J. R., and Clark, R.: Domsiekte or Pregnancy Disease in Sheep.-II. Onderstepoort J. Vet. Sci. & Anim. Indus., *17*, (1941): 245–296.

[8] Hechter, O., Solomon, M. M., Zaffaroni, A., and Pincus, G.: Transformation of Cholesterol Acetate to Adrenal Cortical Hormones. Arch. Biochem. & Biophys., *46*, (1953): 201–214.

[9] Henderson, J. A.: Observations on Reproduction and Associated Conditions in a Herd of Dairy Cattle. Cornell Vet., *28*, (1938): 173–195.

[10] Hewitt, W. F., Jr., and Van Leire, E.: The Question of Thyroid Weight During Pregnancy with Further Observations of Adrenal Weight in Late Pregnancy. Endocrinology, *28*, (1941): 62–64.

[11] Houssay, B. A.: The Influence of Adrenal Insufficiency During Pregnancy on the Mother and on the Offspring. Rev. Soc. argent. biol., *21*, (1945): 316–331.

[12] Ingle, D. J., and Fisher, G. T.: Effects of Adrenalectomy During Gestation on the Size of the Adrenals of Newborn Rats. Proc. Soc. Expti. Biol. & Biophys., *46*, (1953): 201–214.

[13] Krupski, A.: Beiträge zu Physiologie und Pathologie

XUM

des endokrinen Systems. I. Über die Grössenverhältnisse einiger innersekretorischer Drüsen beim Rind, Schaf und Schwein. Schweiz. Arch. f. Tierheilk., *63*, (1921): 419–438.

[14] Long, C. N. H.: The Relation of Cholesterol and Ascorbic Acid to the Secretion of the Adrenal Cortex. Recent Progr. Hormone Res., *1*, (1947): 99–122.

[15] McIntosh, R. A.: Acetonemia-Ketosis. Canad. J. Comp. Med., *8*, (1944): 227–232.

[16] Nicander, L.: Histological and Histochemical Studies on the Adrenal Cortex of Domestic and Laboratory Animals. Acta anat., *14* (suppl. 16), (1952): 1–88.

[17] Popjak, G.: Lipids of the Rat Adrenal in Shock Caused by Experimental Crushing Injury. J. Path. & Bact., *56*, (1944): 485–496.

[18] Randall, L. O., and Graubard, M.: The Adrenal Lipids in Pregnant Rabbits. Am. J. Physiol., *131*, (1940): 291–295.

[19] Reiner, C. B.: The Schultz Histochemical Reaction for Cholesterol Observation on Specificity and Sensitivity. Lab. Invest., *2*, (1953): 140–151.

[20] Saarinen, V. P., and Shaw, J. C.: Studies on Ketosis in Dairy Cattle. XIII. Lipids and Ascorbic Acid in the Liver and Adrenals of Cows with Spontaneous and Fasting Ketosis. J. Dai. Sci., *33*, (1950): 515–525.

[21] Sampson, J.: Ketosis of the Dairy Cow. Ann. Path. Exchange, Univ. of Illinois, 1945 (mimeo.).

[22] Sarason, E. L.: Morphologic Changes in the Rat's Adrenal Cortex Under Various Experimental Conditions. Arch. Path., *35*, (1943): 373–390.

[23] Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H.: The Effect of the Adrenotrophic Hormone of the Anterior Pituitary on the Cholesterol Content of the Adrenals. Yale J. Biol. & Med., *16*, (1944): 361–392.

[24] Sayers, G., Sayers, M. A., Liang, Tsan-Ying, and Long, C. N. H.: The Effect of Pituitary Adrenotrophic Hormone on the Cholesterol and Ascorbic Acid Content of the Adrenal of the Rat and Guinea Pig. Endocrinology, *38*, (1946): 1–9.

[25] Shaw, J. C., Hatziolos, B. C., and Saarinen, V. P.: A Biochemical and Histopathological Study of Ketosis in Dairy Cattle. Abstr. in J. Dai. Sci., *31*, (1948): 667.

[26] Shaw, J. C., Saarinen, V. P., Hatziolos, B. C., and Leffel, E. C.: Biochemical and Histopathological Studies of Fasting Ketosis and Spontaneous Ketosis in Cows. J. Dai. Sci., *31*, (1948): 718.

[27] Snedecor, George W.: Statistical Methods. 4th ed. Iowa State College Press, Ames, 1955.

[28] Verne, Jean, and Herbert, S.: Les types lipidiques de la cortico-currénale chez les mammifères, leur classification et leur signification. Ann. endocrinol., *12*, (1951): 192–198.

[29] Walaas, E., and Walaas, O.: Studies on the Compensatory Hypertrophy of the Fetal Adrenal Glands in the Albino Rat, Produced by Adrenalectomy During Pregnancy. Acta path. et microbiol. scand., *21*, (1944): 640–672.

[30] Weber, A. F., Pritchard, W. R., and Sellers, A. F.: Studies of the Bovine Adrenal Gland. I. The Production of Lipid Accumulation and Other Histological and Cytological Changes in the Zonae Glomerulosae of Calves. Am. J. Vet. Res., *17*, (July, 1956): 402–409.

[31] Weber, A. F., Phillips, M. G., and Bell, J. T., Jr.: An Improved Method for the Schultz Cholesterol Test. J. Histochem. & Cytochem., *4*, (1956): 308–309.

[32] Whitehead, R.: The Fat of the Adrenal Cortex in Fasting Guinea Pigs and Rabbits. J. Path. & Bact., *54*, (1943): 169–176.

[33] Winters, L. M., Green, W. W., and Comstock, R. E.: Prenatal Development of the Bovine. Minnesota Agric. Exper. Sta. Tech. Bull. 151, (1953).

The Comparative Histology of the Skin of Hereford and Aberdeen Angus Cattle

STEVE GOLDSBERRY, M.S., and M. LOIS CALHOUN, D.V.M. Ph.D.

Tuskegee, Alabama, and East Lansing, Michigan

THE NECESSITY of normal skin studies has been given added impetus by the increased interest in veterinary dermatopathology.

REVIEW OF LITERATURE

Trumbower [12] briefly described the sweat glands of the ox in his report on skin diseases of cattle. A histological study on the integument of domestic animals was made by Stoss.[9] Yamane and Ono,[14] using European and Asiatic cattle, investigated the relationship of skin structure to climatic adaptability. They included a thorough review of the literature from 1904 to 1936.

According to Sisson and Grossman,[8] the skin of the ox, which is the thickest of all domestic animals, averages 4 to 5 mm. However, it varies with age, sex, breed, and body region. Trautmann and Fiebiger [12] described the integument of the ox and other domestic animals.

According to Stoss,[9] the dermis of the ox consists of dense collagenous fibers. Dempsey [3] observed that the papillary and reticular layers are not distinctly divided in European cattle. However, the fibers of the papillary layer consist of a loose network of fine elastic and reticular fibers, while those of the reticular layer contain dense collagenous bundles. Skin fat was found to be restricted to the lumbar region. Yamane and Ono [14] reported both layers and observed a transitional zone in the region of the hair bulb which contained both fiber types.

Szodoray [11] found that the dermo-epidermal junction consisted of reticular fibers and amorphous ground substance. Electron micrographs by Maximow and Bloom [6] showed a thin homogeneous basement membrane at this region.

A key for the identification of species of domestic animals, based on hair structure, was given by Trautmann and Fiebiger.[12] Yamane and Ono [14] compared hair population and hair structure of zebu and European cattle.

The structure of the bovine sweat glands was studied by Lowery *et al.*[5] and by Yang and Goodall.[17] Nay and Dowling [7] presented a technique for measuring the size of sweat glands in biopsied bovine skin sections. The nasolabial glands of the bovine animal were examined by Zimmerman.[18] Yang [16] gave an accurate method for assessing skin pigmentation and also reported on the histochemistry of the sweat and sebaceous glands of the ox. Böhm and Hloušek [2] related the amount of, and elastic fibers in, subcutaneous tissue in cattle to skin folds. A review by Beerman and Pillisbury [1] on skin technique, physiology, and histochemistry is a valuable reference.

SECURING AND PROCESSING SPECIMENS

The animals used in this study were from the Hereford and Aberdeen Angus herds of Michigan State University. Two noncastrated males and 2 females of each breed, 14 to 21 months old, were used.

At the time of slaughter, skin specimens were taken from 24 body areas and fixed in 10 per cent formalin for 48 hours. Strips of skin, 2 by 14 mm., were cut parallel to the angle of the hair roots. With sections of this type, a study of the complete hair root and follicle, the various skin layers, the skin glands, and their interrelationships was facilitated. Sections of 10 by 10 mm. were satisfactory for studying horizontal sections.

The dehydration and embedding methods of Johnston *et al.*[4] were followed, and sections were stained with hematoxylin and eosin, and the Weigert-van Gieson stain.

Pigment evaluation (table 3) was determined by a slight modification of Yang's [16] method. Sections were deparaffinized in xylene and mounted without staining.

Using an ocular micrometer, the average thickness of the skin was determined by measuring vertical sections at various locations.

RESULTS AND DISCUSSION

A study of the total skin thickness, as well as the thickness of various layers, indicated certain sex and breed differences. Because of the limited area and specialization of certain regions (table 2), the 13 areas listed (table 1) were considered most typical for an over-all picture. In both the male and female Herefords, the total skin thickness was greater than in the Aberdeen Angus. The male Aberdeen Angus had a thicker skin than the female.

Epidermis.—The five subdivisions of the epidermis described by Trautmann and Fiebiger,[12] the strata cylindricum, spinosum, granulosum, lucidum, and corneum, were present in the perianal region, the

From the Departments of Anatomy, Tuskegee Institute, Tuskegee, Ala., and Michigan State University, East Lansing. Based on a thesis submitted by the senior author in partial fulfilment of the requirements for the M.S. degree at Michigan State University.

TABLE 1—Measurements of Thirteen General Body Areas of Hereford and Aberdeen Angus Breeds

Area	Epidermal thickness (μ)**						Dermal thickness (mm.)**						Total skin thickness (mm.)**					
	Hereford			Aberdeen Angus			Hereford			Aberdeen Angus			Hereford			Aberdeen Angus		
	♂1	♂2	♀1	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2
Ano-anal neck	64	42	70	48	24	45	50	57	7.4	7.0	6.2	8.0*	6.8	5.8	5.0	6.3*	7.5	7.0	6.4	8.1	6.8	5.9	5.0	6.4*
Dorsal thorax	49	72	58	48	16°	49*	84	80	6.4	4.9	5.6	7.0	3.7	5.5	5.7*	6.0	6.7	5.0	5.6	7.0	3.7	5.6	5.8*	6.1
Dorsal lumbar	32	84	56	120*	57*	48	30°	30°	6.2	7.2	3.6°	5.9	6.3	7.1	4.5	5.1	6.2	7.3	3.6°	6.0	6.4	7.1	4.5	5.1
Ventral brisket	55	78	74	72	43	41	58	58	6.0	7.9*	6.0	7.4	5.0	5.0	2.4°	4.0	6.1	8.0*	6.7	7.5	5.0	5.1	2.5°	4.1
Ventral abdomen	75*	96*	98*	54	48	31	144*	103	7.9*	5.7	5.5	6.0	4.4	4.6	3.6	4.4	8.0*	5.8	5.6	6.0	4.4	4.7	3.8	4.5
Ventral axilla	32	48	42	38	50	30	126	145*	7.0	4.7	7.3*	5.5	3.4°	3.7°	2.4°	2.6°	7.0	4.8	7.3*	4.5°	3.4°	3.8°	2.5°	2.7°
Ventral groin	73	36	71	72	47	40	36	33	5.9	6.7	5.7	5.9	5.3	4.0	5.2	4.3	6.0	6.8	5.8	6.0	5.3	4.4	5.3	4.4
Lateral neck	57	30°	37	36	29	33	138	77	7.8	5.9	5.2	5.5	9.1*	7.2*	3.4	5.6	7.8	6.0	5.2	9.5*	9.1*	7.2*	3.5	5.7
Lateral thorax	27*	60	53	48	42	38	60	70	5.3	4.4	6.3	7.0	4.5	5.5	5.2	2.9	5.3	4.5	6.3	7.0	4.5	5.6	5.3	3.0
Lateral abdomen	33	48	53	72	49	45	36	33	4.5	4.0°	6.0	5.4	5.8	5.3	3.0	5.0	4.6*	4.0°	6.1	5.5	5.9	5.3	3.0	5.0
Gluteal region	43	30°	50	30°	42	40	36	40	7.3	4.2	5.5	6.0	4.2	5.6	5.5	6.0	7.4	4.3	6.0	6.0	4.2	5.7	5.5	6.0
Upper foreleg	77	42	26°	66	36	24°	48	35	5.1	4.2	3.7	4.9°	4.0	4.3	5.0	3.2	5.2	4.3	3.7	5.0	4.0	4.6	5.0	3.2
Upper hindleg	65	36	60	60	30	34	42	42	3.8°	6.2	4.5	6.4	4.4	4.4	3.2	5.4	4.6°	6.3	4.6	6.5	4.4	4.4	3.3	5.4
Averages	50.9	54.0	57.5	58.7	39.4	38.3	68.3	61.7	6.2	5.6	5.5	6.2	5.1	5.2	4.2	4.7	6.3	5.7	5.6	6.5	5.2	5.3	4.2	4.7

* Thickest; * thinnest; ** Each value given represents the average of five observations.

hoof margins, and the Achilles insertion only. In most instances, there were only two clearly defined subdivisions—the stratum corneum and the stratum germinativum (fig. 2).

The epidermis was generally found to be thickest in areas where the hair was thin or absent. It was especially thick on the muzzle, where it consisted of 150 to 200 cell layers (fig. 1), and on the hoof margins, perianal region, and horn margins. The epidermis of the male Hereford tended to be thicker than that of the Aberdeen Angus male. The epidermis of the Aberdeen Angus female also exceeded that of the male Aberdeen Angus in thickness. While not so marked, the measurements in the female Hereford were somewhat greater than those in the male.

The thinnest epidermis in the Herefords examined was located laterally on the thorax, neck, gluteal region, and upper forelegs. In 3 of the 4 Aberdeen Angus, the thinnest epidermis was on the dorsal part of the body. If the muzzle is excluded, 3 of the 4 Herefords studied showed the thickest epidermis on the ventral abdominal wall.

The stratum corneum usually consisted of several layers of cornified epithelium which stained pink with hematoxylin and eosin (fig. 1). It was thicker in the Aberdeen Angus.

The stratum lucidum was generally absent. It was present only in the perianal region, the hoof margin, and over the Achilles insertion. The lucidum was usually present on sections which also showed the stratum granulosum.

The stratum granulosum was constant only in the perianal region and occurred occasionally over the extremities. The granular layer consisted of one to three rows of cells. Most of the cells were in a state of degeneration. The nuclei stained palely and, in many cases, the nuclear outline was lost. There were large basophilic droplets in the cytoplasm.

The stratum spinosum consisted of polygonal cells which became spindled near the surface in areas of more than six layers. There was little change in shape in thinner areas. While the intercellular processes were not prominent in areas which consisted of fewer than six cell layers, they were easily demonstrated in areas consisting of more than six cell layers.

The stratum cylindricum consisted of a single layer of columnar cells which varied

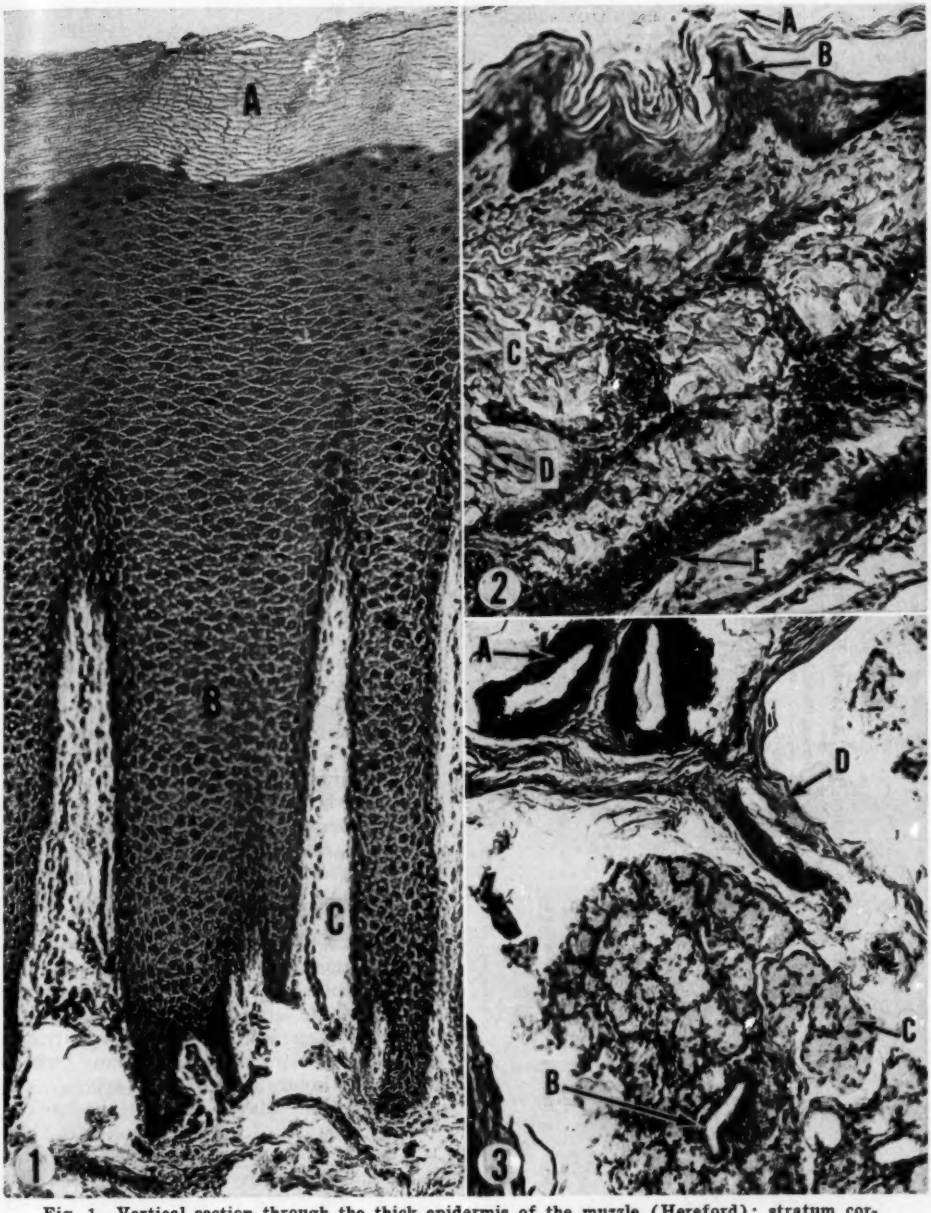


Fig. 1—Vertical section through the thick epidermis of the muzzle (Hereford): stratum corneum (A); stratum germinativum (B); papillary body of the dermis (C). H & E stain; x 140.

Fig. 2—Vertical section through the thin epidermis of the ventral abdomen (Hereford): stratum corneum (A); stratum germinativum (B); stratum papillare of the dermis (C); fine elastic fibers (D); cords of cells typically found in the stratum papillare (E). Weigert's elastic tissue stain; x 160.

Fig. 3—Vertical section through the compound tubular glands found only in the muzzle (Aberdeen Angus): large pigmented excretory ducts (A); intralobular duct (B); cross section of glands (C); collagenous fibers forming septum between lobules (D). H & E stain; x 120.

TABLE 2—Measurements of Nine Specific Body Areas of Hereford and Aberdeen Angus Breeds

Area	Epidermal thickness (μ) *						Dermal thickness (mm.)*						Total skin thickness (mm.)*					
	Hereford			Aberdeen Angus			Hereford			Aberdeen Angus			Hereford			Aberdeen Angus		
	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2
Forehead	54	66	61	54	72	43	100	95	7.7	5.4	4.9	5.6	6.4	8.1	6.9	6.1	7.7	5.5	5.0	5.6	6.5	8.1	7.0	7.0
Tail root	47	36	68	60	115	59	66	52	7.3	8.0	5.7	5.4	4.4	6.1	4.9	6.6	7.3	8.0	5.8	5.5	4.5	6.1	5.0	6.5
Ventral dewlap	50	54	48	48	33	37	84	85	14.9	5.4	6.0	9.5	8.9	6.3	7.4	6.0	15.0	5.5	6.0	9.5	9.0	6.3	7.5	6.1
Ventral udder or scrotum	40	48	70	48	22	32	42	52	6.2	4.5	4.2	5.5	4.0	3.6	5.0	5.0	6.2	4.5	5.0	5.5	4.0	3.6	4.0	5.1
Fetlock	51	66	100	42	38	46	30	41	6.0	4.7	4.0	5.0	3.0	4.0	3.0	3.3	6.0	4.8	4.1	5.0	3.2	4.0	3.0	3.3
Lower hindleg	45	120	127	36	49	30	42	42	3.8	6.2	4.5	6.4	4.4	4.4	3.2	5.4	4.6	6.2	4.6	6.5	4.4	4.4	3.2	5.4
Achilles insertion	46	24	220	36	76	52	108	92	4.4	4.9	6.0	4.5	2.3	4.3	3.0	5.2	3.9	5.9	6.1	4.5	4.3	4.3	3.0	5.2
Perianum	48	64	91	90	280	65	72	76	5.9	3.5	5.5	7.0	4.2	4.0	2.9	4.4	4.4	3.5	5.8	7.0	4.3	4.0	3.0	4.5
Muzzle	763	1,350	339	90	1,650	973	1,100	1,107	Unable to determine margin												Unable to determine margin			

* Each value given represents the average of five observations.

in height with the thickness of the epidermis. They were tallest where the epidermis was thick and cuboidal or polygonal where it was thinnest.

For convenience, the authors use the term "stratum germinativum" to include the active cellular portion of the epidermis. It was thicker in the Hereford than in the Aberdeen Angus. Only a few mitotic figures were present, the majority of which were in the basal layer.

A true basement membrane was not apparent. The basal cells of the epidermis were associated with fibrous processes which extended into the adjacent tissues of the dermis. No definite pattern of attachment in these processes could be established.

A quantitative evaluation of the epidermal pigment revealed noticeable breed differences, but no sex differences. Since the pigment granules were fine and black in the Aberdeen Angus, and those of the Hereford were always brown, color could not be used as an evaluation factor. The pigment appeared to be infiltrated throughout the cytoplasm, and in the areas of greatest density it obstructed the cell outlines (fig. 3). Sunlight appeared to influence the quantity of pigment. The dorsal areas (interscapular, thoracic, and lumbar regions) showed the greatest density of pigment, the lateral areas and the extremities were intermediate, and the ventral and axillary areas showed the least amount (table 3). In the Hereford, the quantity was greater in the red areas than in the white.

Dermis.—The dermis was divided into two layers, the stratum papillare and stratum reticulare. Both layers of the dermis were present, but there was no clearly defined dividing line. There was a transitional zone in the area of the hair bulb in which the layers seemed to overlap.

The papillary layer (fig. 2) consisted of a loose network of interlacing elastic fibers and small collagenous bundles extending from the epidermis to the end of the hair follicle. No breed or sex differences were observable. Connective tissue fibers nearest the epidermis appeared exceptionally small. There was a proportional increase in fiber size with depth. The elastic fibers were more frequent at the insertion of the arrector pili muscle on the hair follicle, but no definite attachment of the fibers to the muscle could be seen.

Another feature of the papillary layer

TABLE 3—Pigment Evaluation

Location	Aberdeen Angus				Hereford			
	♂1	♂2	♀1	♀2	♂1	♂2	♀1	♀2
Forehead	1+B	4+B	2+B	2+B	0—W	0—W	0—W	0—W
Dorsal neck	3+B	2+B	3+B	3+B	0—W	1+R	3+R	1+RW
Dorsal thorax	2+B	3+B	2+B	3+B	0—W	0—W	3+R	3+R
Dorsal lumbar	3+B	3+B	4+B	3+B	2+R	2+R	3+R	3+R
Tail root	2+B	3+B	4+B	4+B	1+R	1+R	3+R	3+R
Ventral dewlap	2+B	1+B	2+B	3+B	0—W	0—W	0—R	0—W
Ventral brisket	1+B	1+B	0—B	1+B	0—W	0—W	0—R	0—W
Ventral abdomen	1+B	1+B	1+B	1+B	0—W	0—W	0—R	0—W
Ventral udder/scrotum	3+B	2+B	0—B	0—B	0—W	3+W	0—R	0—W
Ventral axilla	1+B	1+B	1+B	1+B	0—W	0—W	0—W	1+R
Ventral groin	2+B	1+B	1+B	1+B	0—W	0—W	0—W	0—W
Lateral neck	2+B	2+B	1+B	1+B	0—W	1+R	3+R	1+R
Lateral thorax	2+B	3+B	4+B	2+B	1+R	1+R	3+R	1+R
Lateral abdomen	3+B	3+B	2+B	2+B	1+R	1+R	2+R	2+R
Gluteus	3+B	2+B	2+B	3+B	1+R	3+R	1+R	1+R
Upper foreleg	1+B	1+B	1+B	1+B	1+R	0—RW	1+R	1+R
Fetlock	1+B	1+B	1+B	1+B	0—W	0—RW	1+R	1+R
Upper hindleg	2+B	1+B	1+B	1+B	0—R	0—R	0—W	1+R
Lower hindleg	2+B	2+B	1+B	1+B	1+W	0—W	2+R	0—W
Achilles insertion	3+B	4+B	2+B	1+B	0—W	0—RW	4+R	1+R
Perianum	4+B	2+B	3+B	2+B	2+W	2+R	2+W	3+W
Hoof margin	4+B	4+B	3+B	2+B	0—W	0—W	0—W	1+W
Horn margin	.....	.....	.....	.....	.....	.....	0—W	.....
Muzzle	4+B	4+B	4+B	4+B	2+W	0—W	0—W	0—W

Numbers indicate quantity of epidermal pigment (0 = absence, 1+ = small dispersed patches in basal cells, 2+ = continuous bands with heavier patches, 3+ = moderately dense in several layers of cells, and 4+ = exceptionally dense, cell outline obstructed).

Letters indicate gross color of hair coat (B = black, R = red, W = white, and RW = red and white borders).

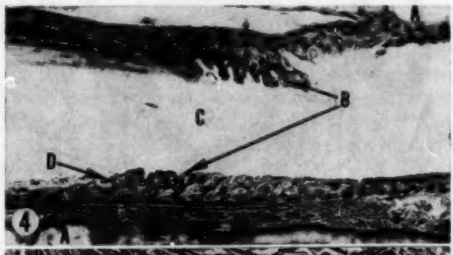
was the clusters and strands of cells (fig. 2) which were invariably found between the connective tissue fibers. The majority of these cells were acidophilic and were similar to the blood eosinophil. Plasmocytes and lymphocytes were also prominent.

The dermal papillae varied considerably with areas of thick or thin epidermis. In the muzzle, with its thickened epidermis, they were highly developed; they were less prominent or absent under thinner epidermis (fig. 1, 2).

All 4 of the Aberdeen Angus cattle had the thinnest dermis in the ventral axillary region. Both the male and female Herefords appeared to have a thicker dermis than did the Aberdeen Angus cattle.

The stratum reticulare consisted of large, coarse, interwoven bundles of collagenous fibers generally parallel to the surface and to each other. Elastic fibers were present only when associated with the supporting areolar tissue, blood vessels, and nerve trunks.

Although all the animals were fattened



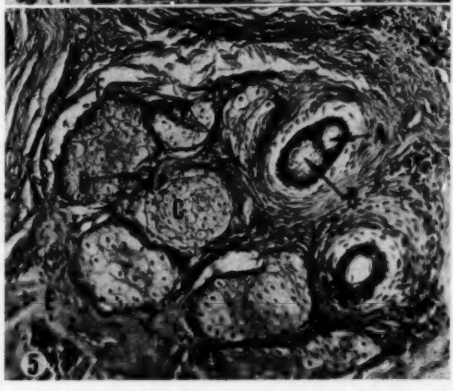


Fig. 4—Longitudinal section through a hair follicle showing the folds which begin near the sebaceous ducts and include the middle third of the follicle (Aberdeen Angus): sebaceous glands (A); folds (B); lumen of hair follicle, hair absent (C); pigment granules (D). H & E stain; x 70.

Fig. 5—Cross section through highly developed sebaceous glands of the perianal region (Hereford): sebaceous duct (A); hair and follicle (B); sebaceous gland (C); sweat duct (D); stratum papillare (E); capillary (F). H & E stain; x 125.

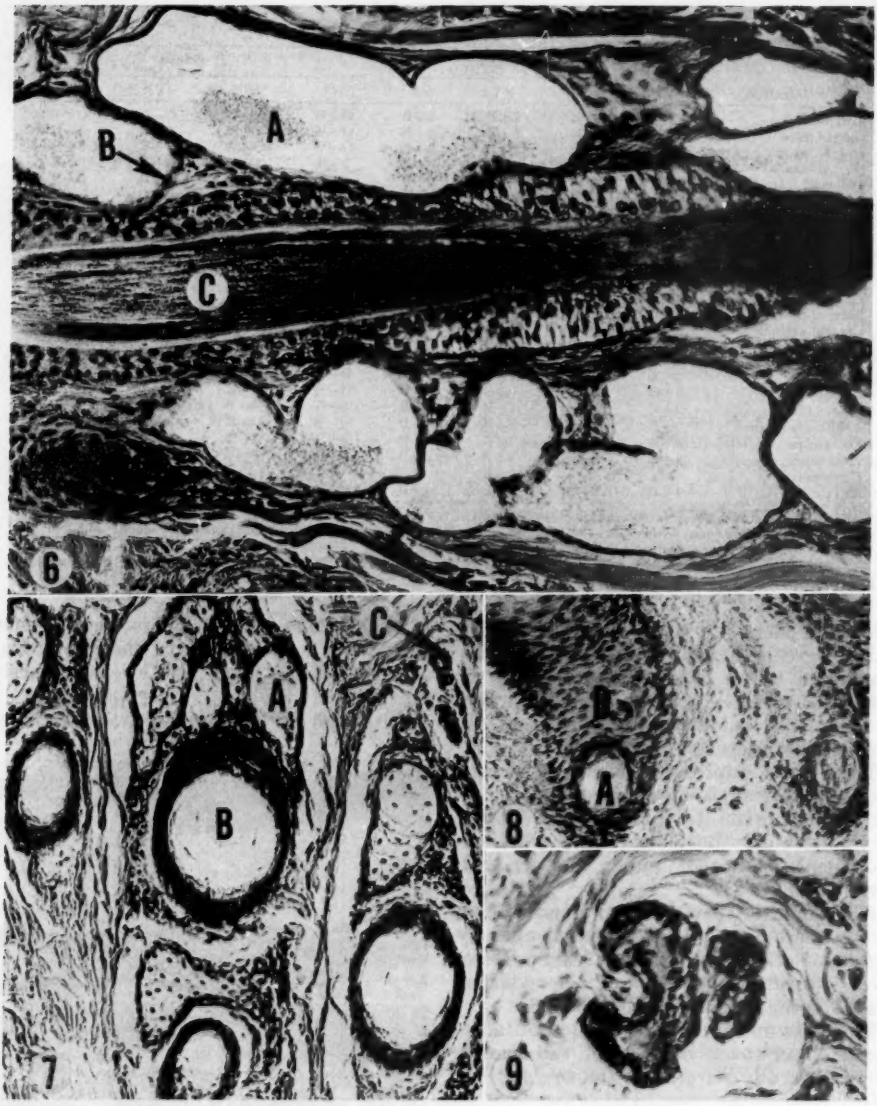


Fig. 6—Longitudinal section through a hair follicle and saccular sweat glands from the dorsal thoracic region (Hereford): lumen of gland containing amorphous material (A); flat cells of the gland wall (B); hair (C). H & E stain; x 170.

Fig. 7—Cross section showing the relation of the sebaceous glands to their respective hair follicle (Aberdeen Angus): lobule of a sebaceous gland near the duct (A); hair follicle (B); small blood vessel (C). H & E stain; x 170.

Fig. 8—Longitudinal section through two epidermal pegs showing hair and sweat duct (Hereford): hair follicle (A); sweat duct (B). H & E stain; x 130.

Fig. 9—Saccular coiled sweat gland in the fetlock (Hereford). H & E stain; x 130.

for slaughter, adipose tissue was absent except in the brisket and in the dewlap, where it was restricted to the deepest portion of the dermis. Large and frequently branching blood vessels and nerve trunks were in the deep portion of the dermis. Large elastic fibers were associated with these vessels. Although numerous nerve trunks were present, no special nerve endings were observed. Three levels of capillary plexuses occurred; one deep in the dermis, one near the hair bulb and sweat glands, and one near the epidermis and sebaceous glands.

Hair.—In both breeds and sexes, a series of distinct folds was invariably present in the wall of the hair follicle (fig. 4). There were usually 15 to 25 folds which began at the opening of the sebaceous ducts and encircled the middle third of the hair. They extended 30 to 40 μ into the lumen of the follicle and occasionally were in contact with the hairs, but no definite attachment mechanism could be seen. They consisted of a homogeneous material which stained pink with eosin but did not take an elastic tissue stain. No cells or fibers were present. A subsequent study of cat skin by Strickland [10] revealed similar structures in the guard hair. He concluded that these were folds of the cuticle of the root sheath.

Hair density apparently varied considerably with the seasons. Since this was not a seasonal study, no conclusions concerning hair density were drawn. The angle at which the hair penetrated the skin was less than 45 degrees except on the udder and anal region, where the angle was approximately 90 degrees. The hair follicle rarely contained more than one hair (fig. 6); when two occurred, one usually showed signs of degeneration.

Sweat Glands.—The sweat glands of both breeds and sexes were divided into three morphological groups: saccular noncoiled, saccular coiled, and compound tubular.

Most of the body was characterized by the saccular noncoiled type which had a large lumen, 300 to 400 μ in diameter. The walls were covered with flat epithelium (fig. 6). The cytoplasm was usually granular and the nuclei were spindle-shaped. The cell outlines were not distinct. The rest of the wall consisted of the spindled myoepithelial cells described by Yang.[15] The lumen often contained a granular amorphous material. The ducts opened into the hair follicle near the surface. They passed between the lobules of the sebaceous gland (fig. 5) on the side of the follicle to which the arrector pili muscle was attached.

The saccular coiled type was located about natural openings in skin margin areas at the point of the hock and on the surface of the fetlock (fig. 9). The ducts opened on the surface epithelium (fig. 8).

The compound tubular glands, found only in the nasolabial region, consisted of branching, closely coiled tubules. The functional epithelial cells were pyramidal, and those of the ducts were cuboidal (fig. 3). The duct system was similar to that of the salivary glands. The duct epithelium was simple cuboidal except in the interlobar ducts, where it was stratified. The stratified squamous epithelium of the large ducts was heavily pigmented in the Aberdeen Angus, but pigment was scant or absent in these ducts in the Hereford.

Sebaceous Gland.—The sebaceous gland structure was similar to that of other species. The size was inversely proportional to the hair density. They were more highly developed in the perianal region (fig. 5) and at the horn, hoof, and muzzle margins but were absent in the hairless areas. While the sebaceous glands might entirely surround the hair follicle, they were most frequently located on one side (fig. 5, 7).

Summary

Sections from 24 body areas on the skins of Hereford and Aberdeen Angus cattle, 2 noncastrated males and 2 females of each breed, were compared histologically.

The results point to certain breed and sex differences in skin thickness; the skins of Herefords of both sexes were thicker than the skins of Aberdeen Angus and that of the male Aberdeen Angus was thicker than in the female. Total skin thickness of the 8 animals varied from 4.2 to 6.3 mm. The stratum corneum was thicker in the Aberdeen Angus, and the stratum germinativum was thicker in the Hereford. The thickest epidermis was on the muzzle and consisted of 150 to 200 epithelial cell layers. The thinnest epidermis was not confined to any particular location. Excluding the muzzle, the thickest epidermis in 3 of the 4 Herefords was on the ventral abdominal wall. There was little evidence of mitotic activity in the epidermis.

An epidermal pigment evaluation revealed breed differences but no sex differences. In the Aberdeen Angus, the pigment

XUM

granules were fine and black; those of the Hereford were coarser and always brown. The dorsal areas showed the greatest density of pigment, the lateral areas and the extremities were intermediate, while the ventral and axillary areas showed the least amount. In the Hereford, the quantity was greater in the red than in the white areas.

A true basement membrane was not demonstrable. A few processes from the basal epithelium extended into the dermis.

The dermis of the Hereford was thicker than that of the Aberdeen Angus. The fibers were also heavier and more densely arranged. The areas studied contained fat only in the loose skin under the neck, or dewlap; the skin over the sternum, or caudal brisket; and the lumbar regions in both species.

A structure not previously described occurred in the hair follicle. A series of 15 to 25 folds began at the opening of the sebaceous duct in the hair follicle. They extended 30 to 40 μ into the lumen and encircled the middle one third of the follicle. Their function has not been determined.

Three types of sweat glands were present: saccular noncoiled in the general body areas, saccular coiled about natural openings and skin margin areas, and compound tubular confined to the nasolabial region.

The sebaceous glands varied in size and form with hair density. In areas where the hair was thin, at the margins of the hooves and around natural openings, they were highly developed. In areas where the hair was dense (neck regions), the hair follicles seemed to crowd the glands into smaller narrow components. In general, they occurred on the same side of their respective hair follicles.

References

[1] Beerman, H., and Pillisbury, D. M.: Dermatopathology as a Field of Research. Lab. Invest., *6*, (1957): 389–411.

[2] Böhm, R., and Hloušek, A.: Prispevek k. poznání podkozního vasiva skotu (Contribution to the Study of Subcutaneous Tissue of Cattle). Sbornik Vysoké Skoly Zemed Brno. B. Spisy Fak. Vet., *1*, (1955): 1–13 (abstr. in Biol. Abstr., *32*, 1958: 438).

[3] Dempsey, M.: The Structure of the Skin and Leather Manufacture. J. Roy. Micros. Soc., *67*, (1948): 21–26.

[4] Johnston, E., Andrews, F. N., and Schrewsbury, C. T.: The Preparation of Muscular Tissue for Histological Study. J. Anim. Sci., *2*, (1943): 244–250.

[5] Lowery, O. H., Gilligan, D. R., and Katusky, E. M.: The Demonstration of Collagen and Elastin in Tissues, with Results Obtained in Various Normal Tissues from Different Species. J. Biol. Chem., *139*, (1941): 795–804.

[6] Maximow, A. A., and Bloom, W. A.: A Textbook of Histology. W. B. Saunders Co., Philadelphia, 1957.

[7] Nay, T., and Dowling, D. F.: Size of Sweat Glands in Shorthorn Strains and Zebu X Shorthorn Cattle. Austral. J. Agric. Res., *8*, (1957): 385–393.

[8] Sisson, S., and Grossman, J. D.: Anatomy of Domestic Animals. W. B. Saunders Co., Philadelphia, 1956.

[9] Stoss, A.: In "Ellenberger's Handbuch der vergleichenden mikroskopischen Anatomie der Haustiere." Paul Parey, Berlin, *1*, (1906): 163–165.

[10] Strickland, J.: The Microscopic Anatomy of the Skin and External Ear of *Felis Domesticus*. Thesis, Michigan State University, East Lansing, 1958.

[11] Szodoray, L.: The Structure of the Junction of the Dermis and Epidermis. Arch. Dermat. & Syph., *23*, (1931): 920–925.

[12] Trautmann, A., and Fiebiger, J.: Fundamentals of Histology of Domestic Animals. Trans. and rev. by R. E. Habel and E. L. Biberstein. Comstock Publishing Associates, Ithaca, N.Y., 1957.

[13] Trumbower, M. R.: Diseases of the Skin of Cattle. Rev. by Leonard Pearson. Special report to the U.S. Department of Agriculture (1904): 320–322.

[14] Yamane, Jinshim, and Ono, Yutaka: Racial Anatomic Investigations of Skin Structures in Water Buffalo, Zebu, Formosan Ox and Holstein-Friesian with Regard to the Problem of Adaptation to Tropical Climates. Trans. by S. Moss. Mem. Fac. Sci. Agric. Taihoku, *3*, (1936): 87–136.

[15] Yang, S. H.: Histochemical Studies of Bovine Sweat Glands. J. Agric. Sci., *42*, (1952): 155–158.

[16] Yang, S. H.: A Method of Assessing Cutaneous Pigmentation of Bovine Skin. J. Agric. Sci., *42*, (1952): 465–467.

[17] Yang, S. H., and Goodall, A. M.: Myoepithelial Cells in Bovine Sweat Glands. J. Agric. Sci., *42*, (1952): 159–161.

[18] Zimmermann, A.: Zur Histologie des Nasenlippen-Spiegels des Rindes. Morph. Jahrb., *74*, (1934): 105–134.

Critical Tests with Polymethylenepiperazine (PMP) Against Parasites of the Horse

J. H. DRUDGE, D.V.M., Sc.D.; S. E. LELAND, Jr., Ph.D.; Z. N. WYANT, A.M.; G. W. ELAM; L. B. HUTZLER, M.S.

Lexington, Kentucky

INVESTIGATIONS on piperazine adipate [1,17] generally have been used in fixing the dosages of the other piperazines for the horse at approximately 40 mg. of piperazine base per pound of body weight. There is evidence [14] that the citrate and phosphate salts are equally effective with the adipate at this dosage level. However, studies on the efficacy of lower dosages have been limited to the early work with the adipate and the recent investigations on the piperazine-carbon disulfide complex. Poynter [15] reported that the latter was highly effective against ascarides at dosages of 25 mg. base/lb.

In the more recent critical tests [3] and field trials [2,4] of the present authors, 20 mg. base/lb. was completely effective against mature and immature ascarides. Small strongyles were also effectively removed by the 20 mg. base/lb.-dose level but the large strongyle, *Strongylus vulgaris*, was not. In the field trials,[4] doses of 10 mg. base/lb. of the complex had a marked removal action on the ascarides but were below the minimal dose for complete removal in suckling foals.

The present trials with polymethylenepiperazine (PMP) [11] were undertaken (1) to provide basic data on its efficacy against the common parasites of the horse for comparison with the other piperazines, and (2) to determine the minimal effective dose against the various types of parasites, especially the ascarides.

MATERIALS AND METHODS

The 13 critical and five subcritical tests that were completed form the bulk of this evaluation. Several additional noncritical studies were made in which pre- and post-treatment egg and larval counts in feces were used to estimate the anthelmintic activity.

Details of the techniques for counting the worms, eggs, and larvae were the same as outlined earlier for similar tests in the horse.[3] In the present critical tests, animals were routinely necropsied at the end of a four-day post-treatment interval.

The animals used for subcritical tests were not necropsied. The anthelmintic action was evaluated by: (1) the number of ascarides voided in the feces after initial treatment, (2) the number voided after retreatment, and (3) weekly eggs per gram (e.p.g.) examinations of feces during a 12-week interval after the last treatment.

The test horses were of mixed light-horse breeding, and all of the parasitic infections were naturally acquired. None of the animals had been given recent anthelmintic treatment. Only young animals (weanlings and yearlings) were used in the critical and subcritical tests. One of the noncritical studies was completed on a group of yearlings, while the others were made on mature mares.

The drug was administered via stomach tube or in the feed. For the former, a suspension of the drug powder in tap water was prepared just before treatment. The grain ration was withheld from the animals on the morning of treatment; there were no other fasting procedures. For administration in the feed, the drug powder was mixed with a day's ration of dampened oats. Varying periods of time up to several hours were required to consume the medicated grain.

In addition to being insoluble in water, PMP is characterized by a relatively high (85%) piperazine base content. To facilitate comparison with the other derivatives, the dosages of PMP are expressed in terms of piperazine base content which is regarded as the anthelmintically active portion. Except for the piperazine-carbon disulfide complex, the activity of these drugs in general has been proportional to the base content.

RESULTS

The critical test data on the five trials with PMP, which were run at dosages of 21, 51, and 106 mg. base/lb. of body weight, are summarized (table 1). Complete removal of both mature and immature ascarides was characteristic of these trials. The removal activity was 0 to 67 per cent against the relatively light infections of mature pinworms. Against the heavy infections of immature pinworms, as en-

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington. Published by permission of the director of the station.

The study was supported in part by grants from the Agricultural Division, American Cyanamid Company, and the Grayson Foundation, Inc.

TABLE 1—Critical Test Data on the Efficacy of Polymethylenepiperazine Against Parasites of the Horse

Horse	Dose			Ascarides (P.e.)		Pinworms (O.e.)		Strongyles			Bots		Stomach worms		Threadworm
	Total Gm.	Mg./lb. (base)	Method	Mature	Immature	Mature	Immature	Small	S.v.	S.ed.	G.i.	G.n.	T.a.	H.m.	S.w.
				NO. VOIDED IN FECES AFTER TREATMENT											
73	8.6	21	Tube	12	13	2	450	1,440	6	0	0	0	x*	x	x
716	13.2	21	Tube	11	0	0	610	9,315	2	0	1	0	x	x	x
77	26.5	51	Tube	1,210	0	1	100	4,870	56	0	0	0	x	x	x
718	52.0	51	Feed	15	3	2	0	11,750	38	0	1	0	x	x	x
719	59.4	106	Tube	0	0	0	645	3,375	28	0	0	0	x	x	x
				NO. RECOVERED AT NECROPSY 4 DAYS AFTER TREATMENT											
73	.....	.....	.....	0	0	1	8,400	250	127	0	59	0	150	90	x
716	.....	.....	.....	0	0	1	4,350	1,100	40	28	80	32	20	10	x
77	.....	.....	.....	0	0	4	1,400	90	74	0	115	2	20	20	x
718	.....	.....	.....	0	0	2	150	50	136	15	389	48	920	0	x
719	.....	.....	.....	0	0	5	4,580	0	32	4	98	13	530	130	5,200
				PERCENTAGE OF EFFICACY											
73	.....	.....	.....	100	100	67	5	85	5	..	0	..	..	..	..
716	.....	.....	.....	100	.....	0	12	89	5	0	1	0	..	..	..
77	.....	.....	.....	100	.....	20	7	98	43	..	0	0	..	..	..
718	.....	.....	.....	100	100	50	0	99	22	0	1	0	..	..	..
719	.....	.....	.....	.....	.....	0	12	100	47	0	0	0	..	..	..

* x = No observation made; P.e. = *Parascaris equorum*; O.e. = *Oxyuris equi*; S.v. = *Strongylus vulgaris*; S.ed. = *Strongylus edentatus*; G.i. = *Gasterophilus intestinalis*; G.n. = *Gasterophilus nasalis*; T.a. = *Trichostrongylus axei*; H.m. = *Habronema muscae*; S.w. = *Strongyloides westeri*.

countered in all but 1 of the animals, removal activity was of a low order—at 0 to 12 per cent. Efficacy of 85 per cent or above against the small strongyles was found throughout this range of doses, but 51 mg. base/lb. was required for removal of 43 to 47 per cent of the large strongyle, *S. vulgaris*. Administration of the drug in the grain ration appeared to reduce the effectiveness against this species in animal 718. No action was found against the other large strongyle, *Strongylus edentatus*, either against the adult worms in the colon, or the immature forms in the subperitoneal cysts. Likewise, no effect on immature *S. vulgaris* in the anterior mesenteric artery was detected. Two species of bots, *Gasterophilus intestinalis* and *Gasterophilus nasalis*, were not removed by the drug. Infections with stomach worms, *Habronema muscae* and *Trichostrongylus axei*, and the threadworm, *Strongyloides westeri*, were found in most of the animals at necropsy.

In the eight critical tests at the lower dosage levels of 5, 10, and 13 mg. base/lb., there was a high order of activity against

TABLE 2—Critical Test Data on Activity of Low Dosage Levels of Polymethylenepiperazine Against Ascarides and Pinworms

Horse	Dose			*Parascaris equorum* Mature			*Parascaris equorum* Immature			*Oxyuris equi* Mature female		
	Total Gm.	Mg./lb. (base)	Method	Removed*	Left†	Efficacy (%)	Removed*	Left†	Efficacy (%)	Removed*	Left†	Efficacy (%)
CRITICAL TESTS												
730	3.4	5	Tube	19	32	37	7	0	100	1	30	3
713	7.2	10	Feed	47	10	83	7	0	100	77	85	48
71	7.8	10	Tube	5	0	100	0	0	.....	0	0	....
72	6.4	10	Tube	32	0	100	16	0	100	20	35	36
78	5.2	10	Tube	1	1	50	2	0	100	2	26	7
631	5.7	10	Tube	6	0	100	3	0	100	6	24	30
662	5.2	10	Tube	45	0	100	64	0	100	0	1	0
710	7.5	13	Tube	82	0	100	0	0	.....	0	0	....
SUBCRITICAL TESTS												
W34	2.7	10	Tube	38	3	93	166	2	99	x**	x	....
W37	3.75	10	Tube	13	1	93	84	0	100	x	x	....
W38	3.4	10	Tube	32	0	100	352	0	100	x	x	....
Y15(1)	7.9	10	Feed	21	26	45	0	0	.....	x	x	....
Y15(2)	12.2	15	Feed	24	1	96	0	0	.....	x	x	....

* No. voided in feces during four-day interval after treatment. † No. found at necropsy in critical tests or removed by later treatment in subcritical tests. ** x = No observation made.

TABLE 3—Worm Egg and Larval Count Data on Critical Test Horses

Horse	Dose mg./lb. (base)	Eggs per gram of feces: Ascarid Before treatment*	Ascarid After treatment†	Ascarid Change (%)	Strongyle Before treatment	Strongyle After treatment	Strongyle Change (%)	Strongyloides Before treatment	Strongyloides After treatment	Strongyloides Change (%)	Larvae per gram of feces: *Strongylus vulgaris* Before treatment	*S. vulgaris* After treatment	*S. vulgaris* Change (%)	Other strongyles Before treatment	Other strongyles After treatment	Other strongyles Change (%)	Strongyloides Before treatment	Strongyloides After treatment	Strongyloides Change (%)
730	5	60	20	—67	2,090	520	—75	0	0	.....	8	12	+50	92	60	—35	0	0	.....
713	10	460	20	—96	2,660	1,990	—25	0	0	.....	x**	x	.....	x	x	.....	x	x	.....
71	10	40	0	—100	1,190	200	—83	0	0	.....	26	20	—23	70	5	—93	0	0	.....
72	10	950	50	—95	1,480	290	—80	0	0	.....	16	27	+69	200	30	—85	0	0	.....
78	10	50	0	—100	2,300	730	—68	20	0	—100	62	35	—44	1,220	120	—90	0	0	.....
631	10	720	60	—92	2,880	580	—80	0	0	.....	x	x	.....	x	x	.....	x	x	.....
662	10	350	80	—77	970	160	—84	0	0	.....	x	x	.....	x	x	.....	x	x	.....
710	13	290	10	—97	575	90	—84	0	0	.....	120	33	—73	340	10	—97	0	0	.....
73	21	150	10	—93	135	60	—56	140	20	—86	33	44	+33	13	0	—100	+	+	.....
716	21	90	5	—97	390	70	—82	0	0	.....	33	21	—36	170	11	—94	0	0	.....
77	51	4,050	280	—93	460	50	—91	0	0	.....	189	33	—83	124	1	—99	0	0	.....
718	51	25	0	—100	390	40	—90	0	0	.....	38	14	—63	325	24	—93	0	0	.....
719	106	0	0	.....	300	0	—100	130	220	+69	47	3	—94	170	1	—99	+	+	.....

* Average of four to six counts during week before treatment. † Average of three counts on fourth day after treatment. ** x = No observation made.

both mature and immature ascarides (table 2). The 10 mg. base/lb. doses were more effective when given via stomach tube than via grain; the 5 mg. base/lb. dose appeared to be too low for effective removal of ascarides. From 3 to 48 per cent of the adult pinworms were removed in this series of horses.

Five additional subcritical trials (table 2), testing the efficacy of the 10 and 15 mg. base/lb. dosages against the ascarid, were generally in accord with the foregoing. Although these animals were not necropsied, the consistent and highly effective removal of the ascarides by the 10 mg. base/lb. dose administered by stomach tube was indicated by (1) the large numbers of both mature and immature ascarides recovered from the feces following the first treatment of each animal; (2) the small numbers of ascarides recovered following retreatment, and (3) the negative ascarid e.p.g. for post-treatment observation periods of 8 weeks (horses W37 and W38) and 3 weeks (horse W34). Administration in the feed seemed less effective, as only 45 per cent were removed by the 10 mg. base/lb. dose in horse Y15. Retreatment of this animal with 15 mg. base/lb. removed all but 1 of the ascarides, and it was eliminated after the third treatment with 20 mg. base/lb.

Worm egg and larval counts were also made on the feces of the critical test horses (table 3). Finding ascarid eggs in the feces of most of the animals on the fourth day after treatment was at variance with the worm recovery data (table 1); it indicated insufficient time for these eggs to pass from the digestive tract, rather than a failure to remove the mature ascarides. Reduction of the strongyle egg count was a consistent finding after treatment, although a 90 per cent or more reduction was not observed below doses of 51 mg. base/lb. This may be accounted for by the more effective removal, at the lower doses, of small strongyles than of large strongyles.

The larval count data is also in accord with this selective action between the large and small strongyles; consistent reduction of *S. vulgaris* larvae was not observed at doses below 51 mg. base/lb., although marked reduction of other strongyle larvae was recorded at the relatively low 10 mg. base/lb. level. The data (table 3) indicate a higher percentage of removal of *S. vulgaris* larvae at the 51 mg. base/lb. dosage levels than of the worms (table 1), which may be

accounted for by a selective removal of the more mature individuals. This phenomenon may also apply to the small strongyles, and be reflected in the generally greater reduction (table 3) of the other strongyle larvae, which were largely composed of small strongyle species, than the worm collection data (table 1) would indicate. Observations of *S. westeri* eggs and larvae failed to establish any drug activity.

One test on a group of 12 yearlings compared the efficacy of two dose levels (20 and 40 mg. base/lb.) against strongyles. The drug was administered by stomach tube. The pre- and post-treatment egg (e.p.g.) and larval (l.p.g.) counts on these animals are summarized (table 4). Both dose levels produced striking reductions in e.p.g. and other strongyle l.p.g. counts, but a consistent and significant reduction in *S. vulgaris* l.p.g. counts was only observed at doses of 40 mg. base/lb.

A similar type of study was made on a group of 5 mares (table 4). The drug was administered in the grain ration, and it was readily consumed. The observations were generally in accord with those on the yearling, except that the 20 mg. base/lb. dosage level reduced the strongyle e.p.g. to a lesser extent while the percentage reduction of *S. vulgaris* larvae was greater.

Two trials were made with PMP in mares for activity of low-level type of administration on strongyles. After 14 daily doses were fed to mare 223 in the grain ration at 5 mg. base/lb. (5.5 Gm. PMP/day), there was no change in the strongyle egg and larval counts. After the same number of doses at twice the rate (10 Gm. PMP/day) were fed to mare 223 in the grain ration at apparent change in the strongyle egg and larval counts. An earlier treatment of this mare with a single dose of PMP at 10 mg. base/lb. in the grain ration effectively removed a light ascarid infection, as determined by the absence of ascarid eggs in the feces during the subsequent two-month period.

DISCUSSION

The highly effective removal of both mature and immature ascarides by PMP at the low dosage level of 10 mg. base/lb. of body weight is of special interest. This is the lowest level based on critical tests that has been reported for any of the piperazines, although there is evidence from field studies [2] that the piperazine-carbon disulfide complex possesses a similar high order of activity against the equine ascarid. Previous critical tests [3] using the complex, in which doses below 20 mg. base/lb. were not tried, resulted in complete removal of *Parascaris equorum*. In the more recent field studies,[2] lower dosages of the complex (10 mg. base/lb.) were consistently effective in markedly reducing or eliminating ascarid eggs in the feces. Piperazine adipate is the only other piperazine on which there are reports of critical tests in the horse [1,7,13] and it appears to be less effective, requiring dosage levels of 40 mg. base/lb. for efficient removal of the ascarid. However, the testing of lower doses of the adipate seemed to be limited to a single trial in a pony that was given 20 mg. base/lb.[1] There is increasing evidence that the generally accepted dosage structure of piperazines for the horse ascarid, based on the early work with the adipate, is not adequate. The dosage and activity relationships among the various piperazine deriva-

TABLE 4—Egg and Larval Count Data on Activity of Polymethylenepiperazine Against Strongyles in Yearlings and Mares

			Eggs per gram of feces			Larvae per gram of feces					
	Dose		Strongyle			*Strongylus vulgaris*			Other strongyles		
No. of animals	Base (mg./lb.)	Method	Before treatment*	After treatment†	Change (%)	Before treatment	After treatment	Change (%)	Before treatment	After treatment	Change (%)
YEARLING EXPERIMENT											
5	20	Tube	1,170	80	−93	9.5	8.3	−13	230	25	−89
5	40	Tube	910	70	−92	14.0	2.4	−83	165	20	−88
2	Control		295	295	0	.....	....	....	.....	....	....
MARE EXPERIMENT											
1	10	Feed	720	520	−28	0.0	2.0	+	505	135	−73
1	20	Feed	530	260	−51	12.5	4.3	−66	245	65	−73
1	40	Feed	470	50	−89	5.4	0.8	−85	110	15	−86
2	Control		820	995	+21	55.0	62.0	+13	190	335	+76

* Average of three counts during five-day pretreatment interval. † Average of three counts during five- to nine-day post-treatment interval.

tives in the horse require further study.

Lack of solubility has been regarded by some workers [5, 9] as favoring *in vivo* activity of piperazines, but this factor does not explain the apparent differences between PMP, the complex, and the adipate, since all three are only slightly soluble in water.

It is also doubtful that the substitution component of PMP is a significant contributing factor to the higher order of activity, because of the general finding that the anthelmintic activity of the piperazines is a function of the base content. The only exception to this has been the complex where the carbon disulfide component, per se an effective agent, undoubtedly contributes to the removal action on *P. equorum*.

Methods of administration played a significant role in determining the dosage level required for effective action. Treatment was more efficient when given by stomach tube than when fed in the grain ration. Although there was no special attempt to compare method of administration and efficacy in the present trials, the limited evidence indicated that the difference was about twofold. Furthermore, this factor probably explains the apparent discrepancy between the lower minimal effective levels found for the removal of *P. equorum* and those reported [6, 8, 10, 12, 16, 17] for most of the piperazines for the removal of *Ascaris lumbricoides* var. suum in swine.

Recent evidence [6] indicates that not only the administration via the ration, but also the practice of treating swine in groups, results in lower efficacy. The stomach tube method, a common procedure in equine practice, offers the opportunity of effecting some economy in using the relatively expensive piperazines for the treatment of ascarid infection in the horse.

The 85 per cent or higher activity in removing small strongyles with dosages of PMP as low as 20 mg. base/lb. is in close agreement with the previous observations on the piperazine-carbon disulfide complex.[3, 4, 14] Therefore, both of these compounds appear to be more active against small strongyles than the adipate,[1] which requires dosages of 40 mg. base/lb. for this level of activity. Lower doses of the PMP were not critically tested, but 10 mg. base/lb. also appears to be effective against small strongyles as determined by reductions in strongyle e.p.g. and larval counts after treatment.

In contrast with the foregoing, larger doses of PMP (40 mg. base/lb.) were required for appreciable action on the large strongyle, *S. vulgaris*. These observations are in agreement with the general evidence on the several piperazines that have been tested, which indicates that this level of piperazine base is the minimal dosage for 50 per cent removal of this species. Increasing the dose did not enhance the action in the present critical tests or in earlier trials with the complex.[3] No activity against *S. edentatus* was detected, which is in keeping with the consistent failure to find action on this species among the piperazines, except from relatively high doses of the complex.[3]

Light infections of adult pinworms were present in the animals given the higher doses of PMP. Removal action attributable to the drug was variable, incomplete, and difficult to evaluate, because of the tendency for these forms to be more or less continually eliminated. However, the activity tended to be less than that reported [1, 3, 13] for other piperazines. Low-grade removal activity against the immature pinworms was characteristic in the present trials and is typical of the findings in other investigations [1, 3, 7, 13] of this group of compounds. The failure to remove more than a small percentage of the large number of immature pinworms that commonly occur in the horse indicates that there is little promise in the piperazines for the successful treatment of this infection, unless a more active preparation or adequate dosing procedure can be found.

SUMMARY

The anthelmintic activity of polymethylenepiperazine (PMP) in the horse was evaluated in a series of 13 critical and 5 subcritical tests. Additional studies, involving 12 yearlings and 7 mares on which pre- and post-treatment egg and larval counts were made, extended the evaluation against strongyles.

The dosage was 5 to 106 mg. of piperazine base per pound of body weight and treatment was via stomach tube, except for several trials on administration via the grain ration. Nearly 100 per cent removal of both mature and immature ascarides resulted from a dosage of 10 mg. base/lb. given by stomach tube. Limited evidence indicated that administration via the grain ration doubled this dosage requirement.

Removal of 85 per cent or more of the

XUM

small strongyles followed doses as low as 21 mg. base/lb. in the critical tests. Data on egg and larval counts of other test animals indicated that 10 mg. base/lb. via stomach tube was also effective against small strongyles. In contrast, there was a significant loss of activity against the small strongyles when this dosage level was fed with the grain ration, even when as many as 14 consecutive daily doses were given.

Removal of approximately 50 per cent of *Strongylus vulgaris* organisms followed dosages of 51 mg. base/lb. and above in the critical tests. Data from the noncritical studies indicated that 40 mg. base/lb. is required for significant removal of *S. vulgaris*.

Activity against pinworms *(Oxyuris equi)* varied from 0 to 67 per cent for the adult forms and 0 to 12 per cent for the fourth-stage larval forms.

No action was found against *Strongylus edentatus, Trichostrongylus axei, Habronema muscae, Strongyloides westeri, Gasterophilus intestinalis,* or *Gasterophilus nasalis.*

References

[1] Downing, W., Kingsbury, P. A., and Sloan, J. E. N.: Critical Tests with Piperazine Adipate in Horses. Vet. Rec., *67,* (1955): 641–644.

[2] Drudge, J. H., and Leland, S. E., Jr.: Critical Tests and Field Studies with Parvex Against Parasites of the Horse. Proc. Third Ann. Meet. Am. A. Equine Practitioners, Dec., 1957.

[3] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., Elam, G. W., Smith, C. E., and Dale, E.: Critical Tests with Piperazine-Carbon Disulfide Complex (Parvex) Against Parasites of the Horse. Am. J. Vet. Res., *18,* (Oct., 1957): 792–797.

[4] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., and Hutzler, L. B.: Field Studies with Piperazine-Carbon Disulfide Complex Against Parasites of the Horse. J.A.V.M.A., *131,* (Sept. 1, 1957): 231–233.

[5] Dunn, T. L.: Effect of Piperazine Derivatives on Certain Intestinal Helminths. Lancet, *268,* (1955).: 592–593.

[6] Enzie, F. D., Wilkens, E. H., and Colglazier, M. L.: The Use of Piperazines as Anthelmintics for Swine. Am. J. Vet. Res., *19,* (Jan., 1958): 19–24.

[7] Gibson, T. E.: Critical Tests of Piperazine Adipate as an Equine Anthelmintic. Brit. Vet. J., *113,* (1957): 90–92.

[8] Guthrie, J. E.: Critical Tests with Piperazine as an Ascaricide in Swine. Vet. Med., *51,* (1956): 235–238.

[9] Hartley, Frank: Activity of Piperazine. Brit. Med. J., *2,* (1955): 205.

[10] Kelley, G. W., Olson, L. S., and Garwood, V.: A Field Evaluation of Ascaricides in Swine. Vet. Med., *51,* (1956): 97–101.

[11] Ladenburg, A., and Herz, W.: Über die Existenz kondensierter Ringe mit Paraverkettung. II. Ber. deutsch. chem. Gesellsch., *30,* (1898): 3043–3045.

[12] Leiper, J. W. G.: The Piperazine Compound V. 19 for the Removal of Ascaris and Oesophagostomum from the Pig. Vet. Rec., *66,* (1954): 596–599.

[13] Poynter, D.: Piperazine Adipate as an Equine Anthelmintic. Vet. Rec., *67,* (1955): 159–163.

[14] Poynter, D.: A Comparative Assessment of the Anthelmintic Activity in Horses of Four Piperazine Compounds. Vet. Rec., *68,* (1956): 291–297.

[15] Poynter, D.: Piperazine-1-Carbodithioic Acid as an Anthelmintic Against *Parascaris Equorum* in Horses. Vet. Rec., *68,* (1956): 429–431.

[16] Riedel, B. B., and Larson, E. J.: The Critical Evaluation of a New Swine Anthelmintic Containing Piperazine and Carbon Disulfide—Parvex. J.A.V.M.A., *129,* (1956): 156–159.

[17] Sloan, J. E. N., Kingsbury, P. A., and Jolly, D. W.: Preliminary Trials with Piperazine Adipate as a Veterinary Anthelmintic. J. Pharmacol., *6,* (1954): 718–724.

The Relationship of Serum Glutamic Oxalacetic Transaminase to Nutritional Muscular Dystrophy in Lambs

KARL F. SWINGLE, Ph.D.; STUART YOUNG, M.R.C.V.S., D.V.S.M.; HUNG CHEN DANG, M.S.

Bozeman, Montana

THE ENZYME glutamic oxalacetic transaminase, which transfers the *alpha*-amino group from aspartic acid to *alpha*-ketoglutaric acid, occurs in most mammalian tissues, being especially abundant in the myocardium, liver, and skeletal muscles. Active destruction of the cells of any of these tissues, from trauma or from disease, releases the transaminase into the blood where it may be determined chemically and the results of such analyses used for diagnostic purposes.[4] Elevated serum glutamic oxalacetic transaminase (SGOT) levels have been reported to accompany progressive muscular dystrophy in human beings.[3,5-8] Similarly elevated SGOT levels were found in clinical cases of naturally occurring muscular dystrophy in lambs in Nevada.[1]

In connection with a study of the cause of muscular dystrophy in calves and lambs in Montana, it was desirable to confirm the subjective clinical diagnosis of the disease by an objective laboratory test. It appeared that the SGOT determination offered the greatest promise of being satisfactory for this purpose. It was hoped that this test would, in addition, provide a sensitive measure of muscle damage in lambs failing to show signs of obvious muscular weakness.

This paper presents data showing the reliability of the SGOT test in the diagnosis of muscular dystrophy in lambs.

MATERIALS AND METHODS

The SGOT tests described were made on serum samples from lambs in a flock maintained at this laboratory for the purpose of experimental work on nutritional muscular dystrophy. In 1957 and 1958, this experimental work was designed to evaluate the effects on the incidence of the disease of various nutrients supplied to the pregnant ewes. The nutritional aspects of this trial will be reported elsewhere.

A total of 418 lambs was born in the flock, and blood samples for SGOT tests were taken routinely from each surviving lamb when 7 and 14 days old. A total of 380 samples taken on the seventh day and a total of 374 samples taken on the fourteenth day were tested. In addition, 366 blood samples were taken from lambs, 3 to 47 days old, for correlation of the SGOT levels with observed signs of illness.

The SGOT was determined by the method of Cabaud *et al.*,[2] in which a buffered aspartic ketoglutaric substrate is incubated with the blood serum, and the oxalacetic acid, formed by the enzymatic deamination of the aspartic acid, is reduced to pyruvic acid. The latter is treated with 2,4-dinitrophenylhydrazine to form a hydrazone, which is extracted with toluene and reacted with alcoholic potassium hydroxide to form a color measurable spectrophotometrically at 490 mμ. The results are expressed as micrograms of pyruvic acid formed per milliliter of serum in 20 minutes at 25 C. These are the SGOT units commonly used in recent clinical literature.

In the course of the experiment, 77 lambs either developed clinical signs of muscular dystrophy or had lesions of the disease at necropsy. The average age at which the first signs became apparent was about 23 days, although close daily observation of signs was not possible under the conditions of the experiment.

RESULTS

Blood Samples Taken at 7 and 14 Days of Age.—The SGOT values for these samples are shown (table 1). Also indicated are the total number of lambs having SGOT test results falling in each 50-unit interval from 50 to 590 units and the number of lambs in each of these intervals that subsequently showed signs or lesions of muscular dystrophy. The figure of incidence is expressed as a percentage of all lambs in each interval.

A direct relationship is shown (table 1) between the amount of transaminase in serum samples taken from lambs 7 or 14 days old and the ensuing incidence of muscular dystrophy in the donor lambs.

From the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating). Montana State College Agricultural Experiment Station paper No. 431, journal series. Mr. Dang is now with the Department of Pharmacology, University of Chicago, Chicago, Ill.

Blood Samples Taken at Other Ages.—Of the 366 samples taken from lambs at various ages, other than 7 and 14 days, 134 gave SGOT test results higher than 400 units. Of these 134 samples, 103 (77%) were derived from lambs that showed clinical signs or lesions of muscular dystrophy at necropsy.

Of the 77 lambs that were classified as having muscular dystrophy, 74 had SGOT levels higher than 400 units in samples taken shortly before or after the recognition of signs of the disease or just before death. The other 3 lambs were not tested within ten days of the time that signs appeared.

Few specific diseases other than muscular dystrophy occurred in the lambs of this flock. However, that a high SGOT level may be associated with other diseases is shown by 1 lamb, affected with suppurative arthritis, which had a consistently high SGOT level for several days before death. Many cases of bronchopneumonia occurred and none of these lambs showed high SGOT levels at any stage in the course of the disease.

DISCUSSION

In evaluating the results of the SGOT tests reported in this flock, only those lambs showing detectable clinical signs or gross necropsy lesions were regarded as having muscular dystrophy. Since the conditions of the experiment did not allow close daily inspection of every lamb, and since only a few of the affected lambs died, it is possible that many mildly affected lambs were not classified as having the disease.

In spite of this possible error, the SGOT results showed a significant relationship to the occurrence of the disease. This relationship was to be expected with samples obtained at ages later than 14 days since, in most cases, these samples were taken from lambs that were already showing signs of the disease. This study confirms the observation of Blincoe and Dye [1] that the SGOT test provides a useful means of supporting a diagnosis of muscular dystrophy.

The relationship that occurs between SGOT levels in samples drawn from lambs 7 or 14 days old and the ensuing incidence of muscular dystrophy in the donor lambs is of special interest. In each case, the donor lamb was clinically normal at the time these samples were drawn. Donor lambs that subsequently showed signs or lesions of muscular dystrophy did not do so until reaching an average age of 23 days. It seems evident from this observation that the SGOT level is a sensitive indicator of early muscular damage and that early lesions of muscular dystrophy may be present for many days before clinical signs become apparent.

One other interesting observation regarding lamb SGOT levels was that all but 20 of the pregnant ewes in this flock were main-

TABLE 1—Serum Transaminase Levels in Lambs 7 and 14 Days Old and Their Relationship to Subsequent Recognition of Muscular Dystrophy in Donor Lambs

Serum transaminase levels (units)	Day 7			Day 14		
	Total No. lambs	No. lambs later showing MD	Incidence of MD (%)	Total No. lambs	No. lambs later showing MD	Incidence of MD (%)
50–99	48	4	8.3	19	0	0.0
100–149	256	41	16.0	175	17	9.7
150–199	42	10	23.8	80	13	16.3
200–249	14	8	57.0	33	11	33.3
250–299	8	6		17	5	
300–349	4	3	69.2	11	6	62.5
350–399	1	0		14	9	
400–449	3	2		6	2	
450–499	0	0		6	3	
500–549	0	0		3	2	
550–599	1	1		2	2	
600–649	1	1		3	2	
650–699	0	0	71.5	2	2	64.0
700–749	0	0		0	0	
750–799	0	0		2	2	
800–849	1	0		0	0	
850–899	0	0		1	1	
900–950	1	1		0	0	
All levels	380	77	20.3	374	77	20.6

MD = muscular dystrophy.

tained through the winter on a basic hay ration obtained from a ranch which has produced dystrophogenic hay consistently for many years. The other 20 ewes were maintained on a hay ration from another ranch where this disease was unknown. These 20 ewes gave birth to 25 lambs, none of which subsequently showed any evidence of the disease, and none of which gave SGOT results above 200 units when 7 or 14 days old. Of all other lambs born in the flock, 9.6 per cent had SGOT levels higher than 200 units at 7 days of age and 28.7 per cent had SGOT levels higher than 200 units at 14 days of age.

Summary

Serum glutamic oxalacetic transaminase tests were made on all surviving lambs when 7 and 14 days old. These lambs were in a flock in which 418 lambs were born and 77 lambs developed clinical signs of muscular dystrophy or showed lesions of the disease at necropsy. These tests were also made on many lambs at other ages.

The results of the tests indicate that a rise in serum glutamic oxalacetic transaminase is a sensitive and early indication of muscular damage in this disease.

References

[1] Blincoe, C., and Dye, W. B.: Serum Transaminase in White Muscle Disease. J. Anim. Sci., *17*, (1958): 224–226.

[2] Cabaud, P., Leeper, R., and Wroblewski, F.: Colorimetric Measurement of Serum Glutamic-Oxalacetic Transaminase. Am. J. Clin. Path., *26*, (1956): 1101–1105.

[3] Dreyfus, J. C., and Shapira, G.: L'Activité Transaminasique du Serum au Cours des Myopathies. Compt. rend. Soc. biol., *149*, (1955): 1934–1935.

[4] Mason, J. H., and Wroblewski, F.: Serum Glutamic Oxalacetic Transaminase Activity in Experimental and Disease States. A Review. Arch. Int. Med., *99*, (1957): 245–252.

[5] Pearson, C. M.: Serum Enzymes in Muscular Dystrophy and Certain Other Muscular and Neuromuscular Diseases. I. Serum Glutamic Oxalacetic Transaminase. New England J. Med., *256*, (1957): 1069–1075.

[6] Sickert, R. G., Fleisher, G. A., and Hanson, M. L.: Serum Glutamic Oxalacetic Transaminase in Certain Neurologic and Muscular Diseases. Proc. Staff Meet. Mayo Clinic, *31*, (1956): 459–464.

[7] Watanabe, R., Kattus, A. A., Semenson, C., and Yamashita, J.: The Serum Transaminase Test in Various Diseases. J. Chron. Dis., *6*, (1957): 561–571.

[8] White, A. A., and Hess, W. C.: Some Alterations in Serum Enzymes in Progressive Muscular Dystrophy. Proc. Soc. Exptl. Biol. & Med., *94*, (1957): 541–544.

Experimental Production of Pneumonia in Lambs

A. H. HAMDY, B.V.Sc., Ph.D., and W. D. POUNDEN, D.V.M., Ph.D.

Wooster, Ohio

IN INVESTIGATING respiratory disease agents in lambs, Pasteurella organisms, pleuropneumonia-like organisms (PPLO), and a virus were isolated.[7, 8] Others also have encountered similar agents in investigating the pneumonia problems of sheep.

Montgomerie *et al.*[13] recovered a gram-negative hemolytic coccobacillus from pneumonic lungs of sheep. The isolated organism was nonpathogenic for sheep by intranasal, intravenous, subcutaneous, or conjunctival routes.

Stamp *et al.*[16] succeeded in transmitting *Pasteurella hemolytica* to sheep by the administration of cultures by different routes.

Downey [4] reported the possible reproduction of pneumonia in 2 of 22 sheep exposed to an agent associated with enzootic pneumonia in New Zealand.

Salisbury [15] found that all transmission tests using cultures of pneumonic lung tissues yielded negative results in sheep.

Greig [5] failed to produce the disease condition in sheep with ovine PPLO. Cordy *et al.* [2, 3] succeeded in experimentally infecting sheep, goats, and pigs with caprine PPLO. A virus possibly related to the psittacosis-lymphogranuloma group has been recovered from lungs of sheep with pneumonic involvement.[11]

Stress has been considered to be among the factors in initiating pneumonia in lambs.[10, 12]

Reports of attempts to produce pneumonia in lambs using combinations of these agents were not encountered. Therefore, our isolates, alone and in combinations, were examined for their potential abilities to produce pneumonic lesions in lambs.

MATERIALS AND METHODS

Forty-one healthy lambs, 4 to 12 weeks old, were used for experimental exposure. They were obtained from the Experiment Station flock and kept in pens in an isolation building. They were allotted to 13 exposure trials. Lambs 1 to 31 were taken from their dams at birth, before nursing, and were raised on cow's milk. The remaining lambs (32 to 41) were raised naturally, but were separated from their dams before the exposure period.

The lambs were inoculated with the agents recovered from pneumonic lesions. The nature of these agents has been described.[7, 8] The inoculum containing the virus was in the form of a 20 per cent suspension of lung tissues, using Difco TC medium 199. The PPLO were propagated in phenol red maltose brcth enriched with 2 per cent Difco serum fraction.

The methods used for detecting the presence of the virus and the other agents were described.[7]

RESULTS

The experimental results are summarized (table 1), including the ages of the lambs, the inoculum used, and the route of administration. Two lambs died; the others were killed for necropsy three weeks after exposure.

Trial 1—Viral Agent.—Four lambs were inoculated with a 20 per cent suspension of mouse lungs infected with the viral agent. Lambs 1 to 3 were inoculated with 4 ml. intratracheally and 5 ml. intranasally, while lamb 4 was inoculated intravenously with 3 ml. No rise in body temperature or evidence of sickness was observed.

At necropsy, lamb 1 showed slight pneumonic lesions which were confined to the apical lobes of the lungs. There were no gross changes in the other 3 lambs, and no bacteria or PPLO were recovered from their lung tissues. Pneumonia was induced in mice by intranasal instillation of a suspension of the lung from lamb 1 only.

When a 20 per cent suspension of the original lamb lung, from which the virus was recovered, was inoculated intratracheally and intranasally into lamb 5 and intravenously into lamb 6, no significant temperature rises were recorded and no signs of pneumonia were observed. The lungs were grossly normal at necropsy. No bacteria or other agents were recovered from the lung tissues by using artificial mediums or mouse inoculations.

Hematological studies of all 6 lambs during the three-week period revealed no change in their erythrocyte, leukocyte, or differential counts, or in their hemoglobin levels.

Trial 2—Pleuropneumonia-like Organ-

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster.

From a dissertation submitted by the senior author to the Graduate School, Ohio State University, Columbus, in partial fulfillment of the requirements for the Ph.D. degree.

TABLE 1—Experimental Exposure of Lambs to Various Agents Recovered from Pneumonic Lesions in Lambs

Trial	Lamb No.	Age (weeks)	Agents	Dosage (ml.)	Route	Observations	
						Clinical	Necropsy
1	1	4	Virus	4, 5	i.t., i.n.	Negative	Slight pneumonia
	2	8	Virus	4, 5	i.t., i.n.	Negative	Negative
	3	8	Virus	4, 5	i.t., i.n.	Negative	Negative
	4	12	Virus	3	i.v.	Negative	Negative
	5	10	Virus	4, 5	i.t., i.n.	Negative	Negative
	6	10	Virus	3	i.v.	Negative	Negative
2	7	8	PPLO	3, 5	i.t., i.n.	Temp. 104.0 F. on day 4	Slight pneumonia
	8	8	PPLO	3, 10	i.t., i.n.	Negative	Negative
	9	8	PPLO	3	i.v.	Negative	Negative
3	10	6	*Past. multocida*	3, 5	i.t., i.n.	Temp. 104.0 F. on day 3	Septicemia, cystitis
	11	6	*Past. multocida*	3, 5	i.t., i.n.	Negative	Negative
	12	6	*Past. hemolytica*	3, 10	i.t., i.n.	Negative	Negative
4	13	5	Stress (environmental)	.....	.....	Negative	Negative
	14	4	Stress (cortisone acetate)	1 Gm.	i.m.	Negative	Negative
5	15	6	Virus, PPLO	3, 5	i.t., i.n.	Negative	Negative
	16	5	Virus, PPLO	3, 5	i.t., i.n.	Negative	Negative
6	32	5	Virus, *Past. multocida*	4, 5	i.t., i.n.	Temp. 105.2 F. on day 4, anorexia	Negative
	33	5	Virus, *Past. multocida*	4, 5	i.t., i.n.	Temp. 104.8 F. on day 3	Negative
7	20	4	Virus, stress	3, 5	i.t., i.n.	Temp. 103.8 F. on days 3 to 5	Pneumonia
	21	10	Virus, stress	3, 5	i.t., i.n.	Temp. 104.8 F. on day 4	Negative
	22	10	Virus, stress	4, 5	i.t., i.n.	Temp. 103.9 on days 3 to 5	Negative
	23	12	Virus, stress	4, 5	i.t., i.n.	Temp. 104.2 F. on day 4	Negative
8	17	8	PPLO, *Past. multocida*	4, 4	i.t., i.n.	Temp. 104.8 F. on day 4	Negative
	18	9	PPLO, *Past. multocida*	4, 4	i.t., i.n.	Temp. 104.4 F. on day 5	Negative
	19	9	PPLO, *Past. multocida*	4, 4	i.t., i.n.	Temp. 105.2 F. on days 3 to 5	Negative
9	24	10	PPLO, stress	3, 5	i.t., i.n.	Temp. 104.1 F. on day 3	Negative
	25	10	PPLO, stress	3, 5	i.t., i.n.	Anemia, leukopenia	Hernia, died
	26	11	PPLO, stress	3, 5	i.t., i.n.	Temp. 104.2 F. on day 4	Negative
10	34	6	*Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 104.4 F. on day 4	Negative
	35	6	*Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 105.6 F. on day 6	Negative
11	27	6	PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 103.8 F. on day 3	Pneumonia
	28	10	PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 105.2 F. on day 4, leukopenia	Pneumonia
	29	6	PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 104.8 F. on day 5	Pneumonia
12	30	6	Virus, PPLO, *Past. multocida*	4, 5	i.t., i.n.	Temp. 105.3 F. on day 3, off feed	Negative
	36	6	Virus, PPLO, *Past. multocida*	4, 5	i.t., i.n.	Temp. 107.0 F. on day 4, leukopenia	Pneumonia
	37	7	Virus, PPLO, *Past. multocida*	4, 5	i.t., i.n.	Temp. 105.8 F. on days 3 to 5	Negative
	38	8	Virus, PPLO, *Past. multocida*	4, 5	i.t., i.n.	Temp. 107.0 F. on day 4, leukopenia	Slight pneumonia
13	31	10	Virus, PPLO, *Past. multocida*, stress	4, 5, 2	i.t., i.n., i.c.	Temp. 104.8 F. on day 4, leukopenia	Pneumonia
	39	7	Virus, PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 107.0 F. on day 5, leukopenia	Pneumonia
	40	8	Virus, PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 105.0 F. on days 4, 5, leukopenia	Pneumonia
	41	6	Virus, PPLO, *Past. multocida*, stress	4, 5	i.t., i.n.	Temp. 106.8 F. on day 5, leukopenia	Pneumonia

i.t. = intratracheal; i.n. = intranasal; i.v. = intravenous; i.m. = intramuscular.

XUM

isms.—Pleuropneumonia-like organism broth cultures were used to inoculate lambs 7 and 8 both intratracheally and intranasally and to inoculate lamb 9 intravenously. No significant temperature rises or clinical signs of pneumonia were observed in either lamb 8 or 9 and no PPLO were recovered from their lung tissues at necropsy. However, lamb 7 showed anorexia and a temperature of 104 F. on the third day and, at necropsy, there were slight pneumonic lesions in the right apical lobe and PPLO were recovered from the lung tissues.

Trial 3—Pasteurella Organisms.—Lambs 10 and 11 were given an 18-hour broth culture of *Pasteurella multocida* intratracheally and intranasally. Lamb 10 had a temperature of 104 F. on postexposure day 3 and was markedly depressed by day 17, when auscultation revealed crepitant sounds in the thorax. Lesions of septicemia, consolidation of the lungs, and cystitis were observed at necropsy. The apical and cardiac lobes of both lungs showed pneumonic lesions and *Past. multocida* was recovered from the lungs. Lamb 11 remained healthy, and no evidence of pneumonia was found at necropsy.

Lamb 12 was inoculated intratracheally and intranasally with a broth culture of *Past. hemolytica.* No signs of illness developed, and pneumonic lesions were not observed at necropsy.

Trial 4—Stress.—Lamb 13 was stressed by altering the environmental conditions, using steam heat (99 F. for 30 min.) followed by chilling (34 F. for 6 hr.). Lamb 14 was stressed by administering a total of 1 Gm. of cortisone acetate intramuscularly over a three-day period. No evidence of illness or rise of temperature was recorded for either animal. Pneumonia was not observed at necropsy, and no bacteria or other agents were recovered from the lung tissues.

Trial 5—Virus and Pleuropneumonia-like Organisms.—Lambs 15 and 16 were inoculated intratracheally and intranasally with a combination of the virus (20% suspension of mouse lungs) and PPLO broth culture. Their body temperatures remained normal and no signs of illness were observed. Hematological examinations revealed no significant changes two, three, four, five, nine, and 14 days after inoculation. Pneumonia was not present in the lungs of either animal, and neither PPLO nor the virus were recovered from the lung tissues by the use of PPLO medium and mouse inoculation.

Trial 6—Virus and Pasteurella Organisms.—Lambs 32 and 33 were inoculated intratracheally and intranasally with equal amounts of a 20 per cent suspension of mouse lungs infected with the virus and *Past. multocida.* Lamb 32 had a temperature of 105.2 F., with anorexia, on the fourth day, while lamb 33 had a temperature of 104.8 F. on the third day. The blood pictures of these 2 lambs were within the normal range. Pneumonia was not observed in either lamb, and the inoculated agents were not recovered from their lung tissues at necropsy.

Trial 7—Virus and Stress.—Lambs 20 to 23 were stressed by giving each a total of 1 Gm. of cortisone acetate intramuscularly over a three-day period. The last injection was given at the time of exposure with the virus material. Lamb 20 had a temperature of 103.6 F. and showed signs of anorexia, depression, and dyspnea on postexposure days 3 to 5, but showed no significant changes in the blood picture during the three-week period. At necropsy, there was pneumonia in the apical lobes of both lungs. Suspension of the lung tissues treated with streptomycin (150 μg./1 ml.) produced pneumonia in mice by intranasal instillation.

Lamb 21 had a temperature of 104.8 F. and anorexia on day 4, but no evidence of pneumonia was found at necropsy. Lamb 22 had a temperature of 103.9 F. on days 3 to 5, but no lesions were found at necropsy. Lamb 23 had a temperature of 104.2 F. on day 4, but no pneumonia was observed at necropsy. No bacteria or other agents were isolated from the lung tissues of these 3 lambs.

Trial 8—Pleuropneumonia-like Organisms and Pasteurella Organisms.—Lambs 17 to 19 were inoculated intratracheally and intranasally with a combination of equal amounts of *Past. multocida* and PPLO. A marked rise of temperature was observed in all 3 on postexposure days 3 to 5 (table 1). There were no changes in the blood picture during the three-week period. The lambs were in good condition when killed and no pneumonic lesions were observed. Pasteurella organisms or PPLO were not recovered from their lung tissues.

Trial 9—Pleuropneumonia-like Organ-

isms and Stress.—Lambs 24 to 26 were stressed physically by altering the environmental conditions, as in trial 4. Each lamb was given PPLO intratracheally and intranasally. Lambs 24 and 26 developed temperatures of 104.0 F. on postexposure day 3 and 104.2 F. on day 4, respectively, but pneumonic lesions were not present in either. Pleuropneumonia-like organisms or other agents were not recovered from their lung tissues.

Lamb 25 developed some nasal discharge, depression, coughing, and anorexia. On day 12, it showed signs of weakness, and had a subnormal temperature (97.0 F.), as well as dyspnea, anemia, and leukopenia. It died on day 14, and necropsy revealed a diaphragmatic hernia with hydrothorax and pneumonia. Pleuropneumonia-like organisms were not recovered from the affected lung tissues.

Trial 10—Pasteurella Organisms and Stress.—Lambs 34 and 35 were stressed with cortisone acetate, as in trial 7, and *Past. multocida* was given intratracheally and intranasally. Lamb 34 developed a temperature of 104.4 F. on postexposure day 4, lamb 35 had a temperature of 105.6 F. on day 6, and both lambs were off feed on these days. No significant changes in their blood pictures were observed during the three-week period. Pneumonic lesions were not present in either lamb at necropsy, and Pasteurella organisms were not recovered from their lung tissues.

Trial 11—Pasteurella Organisms, Pleuropneumonia-like Organisms, and Stress.—Lambs 27 to 29 were inoculated intratracheally and intranasally with a combination of equal amounts of *Past. multocida* and PPLO. Stress was applied by altering the environmental conditions, as in trial 4. Their body temperatures varied from 103.8 to 105.2 F. on postexposure days 3 to 5. They showed signs of anorexia for 24 to 48 hours. No hematological changes were observed in lambs 27 and 29 during the experimental period.

Lamb 28 was weak and unable to stand on day 4. Its leukocyte count dropped from 8,950 before exposure to 4,500 on day 5. Pneumonia was present in both apical lobes of the lungs at necropsy.

When lamb 27 was necropsied, pneumonic lesions were found in only a portion of the right apical lobe but, in lamb 29, the apical and ventral portions of the diaphragmatic lobes of both lungs and the cardiac lobe of the left lung were affected. Pasteurella organisms and PPLO were recovered from the affected lung tissues of these 3 lambs.

Trial 12—Virus, Pleuropneumonia-like Organisms, and Pasteurella Organisms.—Lambs 30, 36, 37, and 38 were inoculated intratracheally and intranasally with a combination of equal quantities of the suspension and broth cultures of the three agents. Lamb 30 had a temperature of 105.3 F. on postexposure day 3 and was weak and depressed with no desire to eat or drink. When it died on day 4, the leukocyte count had dropped from 9,000 to 3,500, and the hemoglobin volume from 16.1 to 6.5 Gm./100 ml. of blood. Necropsy revealed generalized congestion of the lymph nodes, but the lungs were normal. Of the three agents given, only Pasteurella organisms were recovered from the lung tissues.

Lamb 36 had a fever of 107.0 F. on day 4 and of 106.4 F. on day 5. There were signs of dyspnea, anorexia, and weakness during these two days. Leukopenia was recorded on day 7 (2,800), but the blood picture was normal on day 9. Pneumonic lesions were found in both apical lobes at necropsy. Both PPLO and Pasteurella organisms were recovered from the affected areas of the lung tissues, but the virus was not reisolated by mouse inoculation.

Lamb 37 had a temperature of 105.8 F. on days 3 to 5. There were no changes in the blood picture during the three-week period and no pneumonic lesions were found at necropsy. No bacteria or other agents were recovered from the lung tissues.

Lamb 38 had a fever (107.0 F.) of 24 hours' duration and anorexia, dyspnea, and weakness on day 4. The leukocyte count dropped from 10,750 at the time of exposure to 3,350 one week later. Slight pneumonia was present in the right apical lobe of the lungs. Pleuropneumonia-like organisms and Pasteurella organisms, but not virus, were recovered from the lung tissues.

Trial 13—Virus, Pleuropneumonia-like Organisms, Pasteurella Organisms, and Stress.—Lambs 31, 39, 40, and 41 were stressed with cortisone acetate, as in trial 7, and exposed to a combination of three agents, as in trial 12. Lamb 31 had a temperature of 104.8 F. on postexposure

day 4 and leukopenia (2,850) on days 6 and 7. Pneumonia was confined to the apical and cardiac lobes of the lungs. No bacteria or other agents were isolated from the lung tissues.

Lamb 39 had a febrile response of 105.8 to 107.4 F., and showed signs of dyspnea and depression on days 4 to 6. Leukopenia (3,900) was present on days 7 and 9. The leukocyte count returned to normal (9,950) after day 11. Pneumonic lesions were found in the apical lobes of the lungs, and PPLO and Pasteurella organisms were recovered from the affected tissues. Marked pneumonia was induced in mice by intranasal instillation of a 20 per cent suspension of the lung tissues treated with streptomycin (150 μg./ml.).

Lamb 40 developed temperatures of 105 to 106 F., respiratory râles, dyspnea, and depression on days 4 and 5. The leukocyte count dropped to 3,900 on days 7 and 9, but was normal (8,950) on day 11. Pleuropneumonia-like organisms and Pasteurella organisms were recovered from pneumonic lesions at necropsy. Intranasal instillation of a 20 per cent suspension of affected lung tissues, containing 150 μg. of streptomycin per milliliter, into mice resulted in consolidation of the lungs.

Lamb 41 was febrile (maximum of 106.8 F.) from day 3 to 6. Anorexia, dyspnea, respiratory râles, and depression were evident on days 5 and 6. Leukopenia (2,850) of two days' duration was present one week after exposure. Pleuropneumonia-like organisms and Pasteurella organisms, but no virus, were recovered from the pneumonic tissues found in the apical lobes of both lungs.

Thus, of the 41 lambs exposed to these agents, 9 developed clinical signs and lesions of pneumonia. In 3 additional lambs, no clinical signs were observed but slight pneumonic lesions were found. Of these 3 lambs, 1 was inoculated with the virus alone, 1 with PPLO alone, and 1 with a combination of virus, PPLO, and Pasteurella organisms. The 9 with clinical pneumonia were all in the groups given a combination of the agents. These included: 1 of 4 exposed to the virus and stress; all of 3 exposed to PPLO, Pasteurella organisms, and stress; 1 of 4 exposed to virus, PPLO, and Pasteurella organisms; and all of 4 exposed to the three agents with stress.

DISCUSSION

None of the three agents recovered from pneumonic lesions in lambs were able to reproduce the disease in lambs when used singly. Stress also did not reproduce the disease when applied alone. The virus failed to affect 5 out of 6 lambs. The ovine PPLO, alone or with stress, did not produce pneumonia in 4 of 6 lambs (the death of lamb 25 presumably was due to a ruptured diaphragm). These results, which differed from those obtained with caprine and porcine PPLO,[2,17] could be explained on the basis of a difference of pathogenicity of the strains of PPLO.[1,9] Pasteurella organisms were incapable of producing pneumonia. Judging from our experimental results and those of several others (except Stamp *et al.*[16]), *Past. hemolytica* is relatively nonpathogenic for lambs. It may be that Stamp *et al.* were dealing with an unusually virulent Pasteurella infection. Stress, either physical or biological, failed to produce pneumonic lesions in 9 of 11 lambs when applied separately or with one agent. However, when stress was applied with two or all three agents, pneumonia was produced in all of 7 lambs. A combination of any two agents without stress caused a febrile response but no pneumonic lesions in 5 of 7 other lambs.

The factors responsible for pneumonic lesions of the kind we have encountered in lambs probably are of complex nature and involve a number of microbial agents. Physical or biological changes due to weather conditions, nutrition, weaning, castration, docking, or housing may be involved as stress factors. It is possible that the virus or the stress factor provide favorable conditions in which other agents can produce the disease. This view is in agreement with the assumptions of others.[10,12]

SUMMARY

A total of 41 lambs was exposed to a virus, pleuropneumonia-like organisms, and Pasteurella organisms recovered from pneumonic lesions of lambs. None was capable of reproducing pneumonia when used singly.

Pneumonia was not produced by stress when applied alone.

A combination of any two of the three infectious agents resulted in a febrile response in 5 of 7 lambs but no pneumonic lesions.

Combinations of the three microbial agents produced pneumonic lesions in 2 of 4 lambs.

When stress was applied with a combination of two or three agents, all of 7 lambs developed both clinical signs and lesions of pneumonia.

References

[1] Adler, H. E., Yamamoto, R., and Berg, J.: Strain Differences of Pleuropneumonia-like Organisms of Avian Origin. Avian Dis., *1*, (1957): 19–26.

[2] Cordy, D. R., Adler, H. E., and Berg, J.: The Pathogenicity for Swine of a Pleuropneumonia-like Organism from Goats. Cornell Vet., *48*, (1958): 25–30.

[3] Cordy, D. R., Adler, H. E., and Yamamoto, R.: A Pathogenic Pleuropneumonia-like Organism from Goats. Cornell Vet., *45*, (1955): 50–58.

[4] Downey, N. E.: A Preliminary Investigation into the Etiology of Enzootic Pneumonia in Sheep (Southland Pneumonia). New Zealand Vet. J., *5*, (1957): 128–133.

[5] Greig, A. S.: The Isolation of Pleuropneumonia-like Organisms from the Respiratory Tracts of Sheep. Canad. J. Comp. Med. & Vet. Sci., *19*, (1955): 265–271.

[6] Hamdy, A. H.: Observations on Respiratory Disease Agents in Lambs. Dissertation, Ohio State University, Columbus, 1958.

[7] Hamdy, A. H., Pounden, W. D., and Ferguson, L. C.: Microbial Agents Associated with Pneumonia in Lambs. Am. J. Vet. Res., *20*, (Jan., 1959): 87–90.

[8] Hamdy, A. H., and Sanger, V. L.: Characteristics of a Virus Associated with Lamb Pneumonia. Am. J. Vet. Res., *20*, (Jan., 1959): 84–86.

[9] Jungherr, E. L.: Symposium on Chronic Respiratory Diseases of Poultry. IV. The Control of Chronic Respiratory Disease. Am. J. Vet. Res., *19*, (April, 1958): 464–467.

[10] Marsh, H.: The Bacteriology of Progressive Pneumonia of Sheep. J.A.V.M.A., *64*, (Dec., 1923): 304–317.

[11] McKercher, D. G.: A Virus Possibly Related to Psittacosis-Lymphogranuloma Pneumonitis Group Causing a Pneumonia in Sheep. Science, *115*, (1952): 116–118.

[12] Miller, N. J.: Diseases Incident to the Fattening of Lambs. J.A.V.M.A., *96*, (Jan., 1940): 24–29.

[13] Montgomerie, R. F., Bosworth, T. J., and Clover, R. E.: Enzootic Pneumonia in Sheep. J. Comp. Path. & Therap., *51*, (1938): 87–107.

[14] Moulton, J. E., Boidin, A. G., and Rhode, E. A.: A Pathogenic Pleuropneumonia-like Organism from a Calf. J.A.V.M.A., *129*, (Oct. 15, 1956): 364–367.

[15] Salisbury, R. M.: Enzootic Pneumonia of Sheep in New Zealand. New Zealand Vet. J., *6*, (1957): 124–127.

[16] Stamp, J. T., Wett, J. A. A., and Thomlinson, J. R.: *Pasteurella Hemolytica* Septicemia of Lambs. J. Comp. Path. & Therap., *65*, (1955): 183–196.

[17] Switzer, W. P.: Studies on Infectious Atrophic Rhinitis of Swine. II. Intraperitoneal and Intranasal Inoculation of Growing Pigs with a Filterable Agent Isolated from Nasal Mucosa of Swine. Vet. Med., *96*, (1953): 392–394.

Characteristics of a Virus Associated with Lamb Pneumonia

A. H. HAMDY, B.V.Sc., Ph.D., and V. L. SANGER, D.V.M., M.Sc.

Wooster, Ohio

SEVERAL INVESTIGATORS have reported that a virus might be the initial stimulus in pneumonia complexes in lambs.[1,7,10] In a previous paper, it was reported that a virus was recovered from pneumonic lesions in a lamb at slaughter.[4] This investigation was designed to obtain additional information on the isolated agent.

MATERIALS AND METHODS

Trypsinized kidney cell cultures were used for the cultivation of the virus. The method of preparation described by Melnick was followed.[6]

Kidneys, obtained aseptically from a freshly slaughtered lamb, were decapsulated and the cortices were removed. The tissues were minced in a 40-ml. centrifuge tube with scissors and transferred to a pinched Erlenmeyer trypsinization flask. The tissues were washed three times with Hanks' balanced salt solution.[11] After two, three-hour periods of trypsinization with 0.25 per cent trypsin, the suspension was poured through a layer of sterile gauze to remove tissue fragments. The excess trypsin was removed by centrifuging at 600 r.p.m. for five minutes and repeating this procedure after resuspending in Hanks' balanced salt solution.

Finally, the packed cells were suspended in 150 volumes of lactalbumin hydrolysate medium and bovine serum was added to make a final concentration of 10 per cent. The pH was adjusted to between 7.2 and 7.4, using 3 per cent sodium bicarbonate. The viability and the approximate concentration of the cells were determined by examination of smears stained with trypan blue and crystal violet.

Using an automatic pipette, 2 ml. of the kidney cell suspension was inoculated into sterile tubes containing cover slips. They were placed in stationary racks and incubated at 37 C. The tubes were examined daily and the medium was changed when the pH dropped below 7, as determined by the phenol red indicator. After five days' incubation, the medium was changed to lactalbumin hydrolysate medium containing 3 per cent bovine serum. One tenth milliliter of a 10 per cent suspension of mouse lung, infected with the virus in Difco TC medium 199, was added to each tube. Mice were inoculated intranasally with the same virus. This material was also streaked on blood agar plates to check bacterial contamination. Each day, the tubes were examined microscopically (x 100) to determine if cytopathogenic changes had occurred. After three and seven days' incubation, the cover glass cultures were fixed and stained with May-Grünwald-Giemsa stain.[5]

The range of pathogenicity and properties of the virus were also investigated. Embryonating chicken eggs, mice, hamsters, guinea pigs, rabbits, turkeys, and lambs were used. The ninth egg passage was used for measuring the 50 per cent end point *in ovo*.[9] The eggs were candled daily and the yolk material was examined for elementary bodies.[6] The day of death was recorded and egg fluid was checked for contamination, using blood agar plates.

Three-week-old mice, in groups of 12, were lightly anesthetized and 3 drops of a 10 per cent suspension of the mouse lung was instilled intranasally. One half the inoculated mice was killed after 10 days, while the other half was left to determine if and when death occurred.

Serial passages in mice were continued for 20 passages. Smears from consolidated mouse lung were examined for elementary bodies.[6] This virus was inoculated intracerebrally in mice, using 0.03 ml. of a 10 per cent suspension of mouse lung. One tenth milliliter of a similar suspension was inoculated intratracheally in 2 guinea pigs. After their temperatures were taken daily for a period of ten days, they were killed.

Six hamsters, lightly anesthetized, were instilled intranasally with 3 drops of a 10 per cent suspension and killed after ten days. One tenth milliliter of the same suspension was inoculated intranasally in 2 rabbits, 1 of which was killed after ten days and 1 at two weeks.

Turkey poults were inoculated intraperitoneally with 0.5 ml. of the same suspension. After five weeks, their blood was collected for serological studies.

Four lambs were used for experimental reproduction of pneumonia.[8] Filterability of this virus through various sterilizing filters (Seitz and Selas No. 03, 02, 015, and 01) was also included.

RESULTS

The virus was successfully propagated in tissue culture. This was indicated by the

---

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster. Taken from a dissertation submitted by the senior author to the Graduate School, Ohio State University, Columbus, in partial fulfillment of the requirement for the Ph.D. degree.

The authors acknowledge the assistance of Drs. E. H. Bohl and B. B. Hancock, from the Department of Veterinary Bacteriology, Ohio State University, and also thank Dr. R. E. Kissling and Miss M. C. Calvin, from the Communicable Disease Center, Laboratory Branch, Virus and Rickettsia Section, Montgomery, Ala., for the serological examinations.

presence of inclusion bodies in the epithelial cells and the ability to reproduce pneumonia in mice by intranasal instillation of the tissue culture preparation. The virus did not produce any cytopathogenic effects in lamb kidney epithelial cells three or seven days after incubation. However, intracytoplasmic inclusion bodies were demmonstrated seven days after adding the virus (fig. 1). No inclusion bodies or other changes were observed in the control group (fig. 2). Small circumscribed red refractile bodies were found in the smears of the mouse lung stained by Macchiavello's method.[6]

The range of pathogenicity of the virus was determined in various laboratory animals. Three-week-old mice showed signs of sickness, including labored breathing, hunched posture, dullness, and depression. The mortality was low in the early mouse passage but, as the serial passage increased, most of the inoculated mice died 12 to 18 days after exposure. Consolidation of the lungs was frequently observed and the entire lobes were sometimes affected. A few mice showed signs similar to those of encephalitis when 0.03 ml. of the suspension was inoculated intracerebrally.

The guinea pigs had temperatures of 103.5 to 104.2 F. three days after inoculation. Evidence of pneumonia was not observed on necropsy in either. No elementary bodies were demonstrated in smears from their lung tissues. One rabbit showed a few hemorrhagic spots on the lungs when killed after ten days. The other rabbit and the hamsters showed no abnormalities on necropsy. No elementary bodies were demonstrated in smears from their lungs.

The chicken embryos died three to nine days after yolk sac inoculation. The mortality was 100 per cent after the eighth egg passage. The l.d.$_{50}$ in eggs was then found to be $10^{-2.37}$. The dead embryos were hemorrhagic and the yolk was thin and watery. Elementary bodies were also observed in smears from the infected yolk sac stained by Macchiavello's method.[6]

Blood samples were taken and the poults were killed five weeks after inoculation. No lesions were observed. The pooled serums were submitted to the Virus and Rickettsia Laboratory Section of the Communicable Disease Center, Montgomery, Ala. The direct complement-fixation test gave a titer of 1:64 for psittacosis.

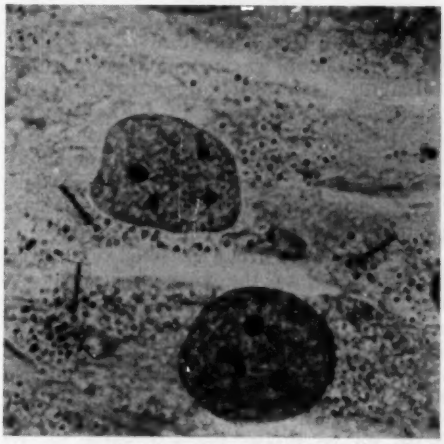


Fig. 1—Lamb kidney epithelial cells in tissue culture, seven days after addition of the virus, showing intracytoplasmic inclusion bodies (arrows). May-Grünwald-Giemsa stain; x 910.

The criteria used for determining the filterability of the virus were the ability to kill the embryonating egg at three to nine days and the ability to induce pneumonia in mice. It was found that the virus from the infected mouse lung was capable of passing through Seitz sterilizing pads and Selas No. 01, 015, and 02 filters. The virus was retained by Selas No. 03 filters at a negative pressure of 50 to 60 mm. of mercury.

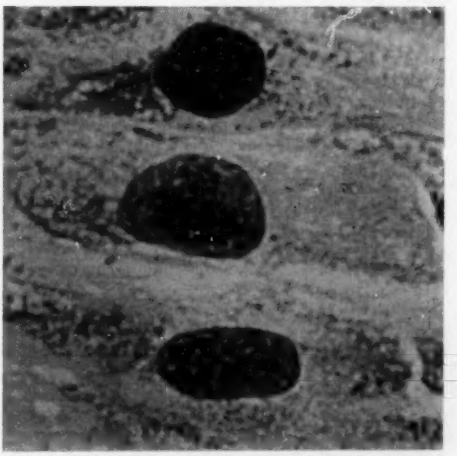


Fig. 2—Normal lamb kidney epithelial cells after 12 days in tissue culture. May-Grünwald-Giemsa stain; x 873.

XUM

DISCUSSION

The virus recovered from a pneumonic lung of a lamb was propagated in tissue culture. Although no cytopathogenic changes were observed in tissue culture, evidence of the viral multiplication was supported by the finding of inclusion bodies and the production of pneumonia in mice by exposing them to the tissue culture preparation.

Serial passage in mice seemed to increase the virulence of the virus for mice. At the twelfth passage, most of the inoculated mice died 12 to 18 days after inoculation. The virus was highly virulent for chicken embryos. Mortality was regularly produced after yolk sac inoculation. Passage of the virus through Seitz sterilizing pads and Selas No. 02 filters, but not Selas No. 03 filters, suggests that it is of a relatively large size. These findings, as well as the report from the Virus and Rickettsia Laboratory, indicate that the recovered agent may be related to the psittacosis group of viruses. This is in general agreement with the reports of other investigators.[2,7,10]

SUMMARY

1) A virus was recovered from pneumonic lesions in a lamb at slaughter. The virus was propagated in ovine kidney tissue culture cells. No cytopathogenic changes were seen, but intracytoplasmic inclusion bodies developed by the seventh day.

2) The virus produced pneumonia in mice when instilled intranasally.

3) Chicken embryos were susceptible to the virus and died within three to nine days.

4) The virus passed through Seitz sterilizing pads and Selas No. 02 filters, but was retained by Selas No. 03 filters.

5) Antibodies against psittacosis virus were demonstrated in turkey serum inoculated with the virus.

References

[1] Bosworth, T. J., and Lovell, R.: The Occurrence of Hemolytic Coccobaccilli in the Nose of Normal Sheep and Cattle. J. Comp. Path. & Therap., *54*, (1944): 168–171.

[2] Gerloff, R. K., and Lackman, D. B.: Report of Wide Distribution of Psittacosis Antibody. Pub. Health Rep., *69*, (1954): 188–189.

[3] Hamdy, A. H., and Pounden, W. D.: Experimental Production of Pneumonia in Lambs. Am. J. Vet. Res., *19*, (Oct., 1958): In press.

[4] Hamdy, A. H., Pounden, W. D., and Ferguson, L. C.: Microbial Agents Associated with Pneumonia in Slaughtered Lambs. Am. J. Vet. Res., *19*, (Oct., 1958): In press.

[5] Jacobson, W., and Webb, M.: The Two Types of Nucleic Acid During Mitosis. J. Physiol., *112*, (1951): 2.

[6] Macchiavello, A.: Diagnostic Procedures for Virus and Rickettsial Diseases. 2nd ed. American Public Health Association Press, New York, N.Y. (1956): 420.

[7] McKercher, D. G.: A Virus Possibly Related to Psittacosis-Lymphogranuloma Pneumonitis Group Causing a Pneumonia in Sheep. Science, *115*, (1952): 543–544.

[8] Melnick, J. L.: Diagnostic Procedures for Virus and Rickettsial Diseases. 2nd ed. American Public Health Association Press, New York, N.Y. (1956): 116–118.

[9] Reed, L. F., and Muench, H.: A Simplified Method of Estimating Fifty Percent End Points. Am. J. Hyg., *27*, (1938): 69–80.

[10] Saito, Y., Schmida, F., Nosu, S., Toda, M., and Omanishi, N.: Studies on the Non-bacterial and Infectious Pneumonia of Goats. I. Isolation and Morphological Findings of the Causative Agent. Kitasato Arch. Exptl. Med., *27*, (1954): 103–114.

[11] Waller, T. H., Enders, J. F., Rubbins, F. C., and Stoddard, M. G.: Studies on the Cultivation of Poliomyelitis Virus in Tissue Cultures. I. The Propagation of Poliomyelitis in Suspended Cell Cultures of Various Human Tissues. J. Immunol., *69*, (1952): 645–671.

XUM

Microbial Agents Associated with Pneumonia in Slaughtered Lambs

A. H. HAMDY, B.V.Sc., Ph.D.; W. D. POUNDEN, D.V.M., Ph.D.;
L. C. FERGUSON, D.V.M., Ph.D.

Wooster, Ohio

PULMONARY LESIONS are readily demonstrated in many lambs at the time of slaughter.[19] Of 995 lambs examined, 44 per cent had various degrees of pneumonic involvement.[11] This report concerns the microbial agents recovered from the lesions in slaughtered lambs and the existence of Pasteurella species in nursing lambs and their dams.

REVIEW OF LITERATURE

Many investigators have incriminated Pasteurella organisms as the causative agent of pneumonia of sheep.[2, 5, 6, 7] Newsom and Cross[18] described a shipping fever type of pasteurellosis in sheep in which pneumonia was the main cause of death. Working on the bacteriology of pneumonia in slaughtered lambs, Spray[22] frequently isolated Pasteurella organisms. Marsh[14] considered that nearly all pneumonia in sheep was caused by Pasteurella infection. Stamp *et al.*[20] described an acute disease of sheep associated with Pasteurella organisms. McGowan *et al.*,[15] working with pneumonia in California lambs, recovered *Corynebacterium pyogenes* in the heart blood of 13 and *Pasteurella multocida* from the lungs of 10 of 50 lambs. They also isolated micrococci, streptococci, and Pseudomonas organisms from affected lungs. Dealing with enzootic pneumonia of sheep in New Zealand, Salisbury[21] found that Pasteurella-like organisms usually were present in pneumonic lungs.

Members of the pleuropneumonia group of microorganisms are known to be associated with respiratory diseases of various species of animals. Greig,[9] in Canada, reported the first isolation of pleuropneumonia-like organisms (PPLO) from the respiratory tracts of sheep. Longley[12] recognized a form of pleuropneumonia in goats that was caused by PPLO. Cordy *et al.*[4] described a highly fatal disease in dairy goats caused by PPLO. Experimentally infected goats, sheep, and pigs showed signs of PPLO infections similar to those in animals with field cases, including arthritis and septicemia.

The absence of bacteria of known etiological significance in many pneumonic lungs of sheep suggested that a virus might be involved in such cases.[1, 7] McKercher[16] reported that a virus, possibly related to the psittacosis-lymphogranuloma-pneumonitis group, was isolated from pneumonic lungs of sheep. Gerloff and Lackman[8] reported that 16 of 473 sheep, tested during a Q fever survey, possessed antibodies against psittacosis-lymphogranuloma virus.

MATERIALS AND METHODS

Bovine blood agar plates were used for bacteriological examinations. Cotton swabs, moistened with sterile buffered saline solution, were used to collect specimens from the laryngeal regions of the lambs and their dams. Milk from the lactating ewes was collected aseptically for cultures. Specimens of lung tissue were taken aseptically from the lungs of lambs at the time of slaughter. Each specimen was inoculated on the blood agar plates. The plates were incubated at 37 C. for 24 hours and examined for colonies suggestive of Pasteurella. Final identification was based on Gram's stain and biochemical reactions.

Morton's liquid medium[17] was used for the isolation of PPLO. Sterile cotton swabs that had been inserted into the nostrils of nursing lambs or portions of lung tissues taken from the lambs at slaughter were introduced into tubes of this medium. After two to seven days' incubation, a loopful from each of the inoculated tubes was streaked on PPLO agar plates (Difco) containing 2 per cent serum fraction. Microscopic examinations (x 100) of the inoculated plates were made after two to seven days.

Pneumonic lung tissues, obtained at slaughter from 8 lambs, were examined for the presence of viral agents. The tissues were minced separately in tissue grinders to make 10 per cent suspensions, using TC medium 199 (Difco). The suspensions were centrifuged at 2,000 r.p.m. for ten minutes and the supernatant fluids were aspirated. Three drops from each of these fluids was instilled intranasally into lightly anesthetized Swiss mice. The inoculated mice were killed after ten days. Serial passages were made until the twentieth transfer had been accomplished. As a control measure, concurrent serial passages were made with normal mouse lung tissues, to investigate the possibility of an agent being present in the mice used in

From the Veterinary Science Department, Ohio Agricultural Experiment Station, Wooster. Dr. Ferguson is now with the Department of Microbiology and Public Health, Michigan State University, East Lansing.

From a dissertation submitted by the senior author to the Graduate School, Ohio State University, Columbus, in partial fulfillment of the requirements for the Ph.D. degree.

The authors thank Dr. D. G. McKercher, School of Veterinary Medicine, University of California, Davis, for his helpful suggestions.

the serial passages. Impression smears were made from the consolidated mouse lungs and stained by Macchiavello's method.[13]

RESULTS

Pasteurella organisms were isolated from the throat swabs of 28 of 41 lambs and 23 of 36 dams (table 1). *Pasteurella hemolytica* was the predominant organism in 25 lambs and 18 dams. This organism formed acid with no gas in lactose, glucose, maltose, mannitol, and sucrose. Cultures of *Past. multocida* were isolated from the other 3 lambs and 5 dams. This agent fermented glucose, mannitol, and sucrose with no gas. However, it did not ferment lactose and maltose and did not produce hemolysis on blood agar plates.

TABLE 1—Isolation of Pasteurella Organisms from Throat Swabs of Lambs and from Throat Swabs and Milk Samples of Their Dams

No. examined		Isolations of Pasteurella from		
Lambs	Dams	Lamb throat	Dam throat	Dam milk
41	36	28	23	2
Percentage		68.3	63.8	7.1

It was noticed that nearly all Pasteurella organisms isolated from the lambs were morphologically and biochemically similar to those from their dams. When the milk samples were examined, *Past. multocida* was found in only two of 36 samples. Both infections were accompanied by acute mastitis and systemic disturbances, with a fever ranging up to 106.2 F.

Of the 41 lambs examined at slaughter, 14 showed various degrees of pneumonia. Approximately 71 per cent of the pneumonic lambs harbored Pasteurella organisms in their throats during their early lives.

When lung tissues from 51 lambs with pulmonary involvement were examined, including the 14 pneumonic lambs mentioned earlier, *Past. multocida* was recovered from 15 lambs, *Past. hemolytica* from 9, streptococci in mixed culture with micrococci from 7, staphylococci from 3, corynebacteria from 2, and Pseudomonas from 5. Pleuropneumonia-like organisms were isolated from pneumonic lesions of 2 lambs. Characteristic PPLO colonies appeared on PPLO agar plates after two to three days' incubation (fig. 1).

A viral agent was recovered from pneumonic lesions in 1 of 8 additional lambs. This agent induced pneumonia in 3-week-old mice when inoculated by intranasal instillation. It killed chicken embryos three to nine days after yolk sac inoculations and produced inclusion bodies in ovine kidney tissue culture cells. Additional characteristics of the viral agent will be described in another report.

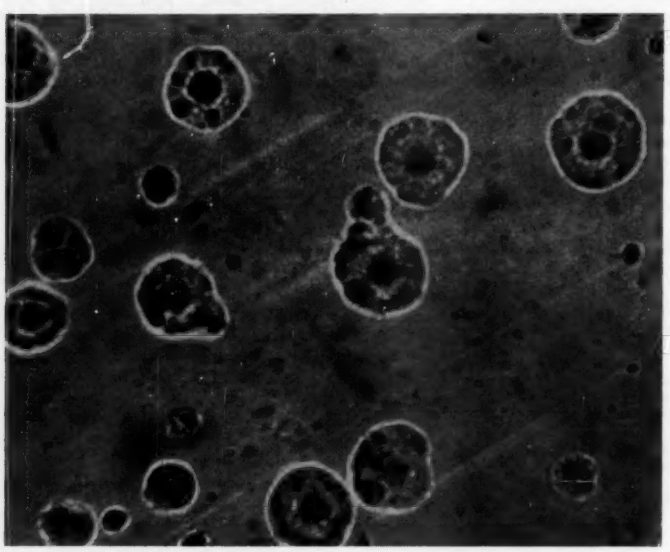


Fig. 1—Colonies of ovine PPLO on PPLO agar plate after three days' incubation, showing the typical dense central zone that appeared to grow down in the medium and a lighter peripheral surface growth giving the appearance of a fried egg. Transmitted light; x 120.

Discussion

It is obvious from the results that the pulmonary lesions in slaughtered lambs are not due to a single agent. Pasteurella organisms, PPLO, and a viral agent were found in this pneumonia complex in lambs.

Pasteurella organisms were frequently recovered from apparently healthy lambs, indicating that they were harboring these organisms in their throat regions during the nursing period. Since the organisms appeared morphologically and biochemically similar to those recovered from the corresponding dams, it may be concluded that the lambs acquired the organisms from their dams. Pasteurella organisms harbored in a lamb's throat may in turn provide the source of mastitis infection. In our work, mastitis was observed in 2 ewes, and *Past. multocida* was isolated from the milk samples and throat swabs from both the ewes and their lambs.

Organisms of the pleuropneumonia group have been associated with respiratory diseases in various species of animals and birds. This ubiquity of PPLO permits some speculation as to whether they are primary pathogens or secondary invaders in pneumonia complexes in lambs. The only report found on the recovery of PPLO from the respiratory tracts of sheep was that made in Canada.[9] In our study, PPLO were isolated from the lung tissues and respiratory tracts of pneumonic lambs. Inoculation of lambs with pure cultures of this ovine PPLO failed to reproduce pneumonia.[11] According to Carter and Schroder,[3] PPLO are probably secondary invaders in virus pneumonia in pigs. Therefore, some skepticism exists in regard to the actual importance of PPLO as a pathogen.

The virus recovered from these lambs produced consolidation in the lungs of mice inoculated by intranasal instillation. The possibility that this agent plays a role in the pneumonia complexes in lambs is further supported by the demonstration of elementary bodies both in smears from the mouse lungs and in tissue cultures and by its ability to kill the developing chicken embryos. This finding is in general agreement with other reports.[16, 20]

Summary and Conclusions

Pasteurella species were isolated from the throats of 68.3 per cent of 41 apparently healthy lambs and 63.8 per cent of their dams. At slaughter, pneumonic lesions were found in 14 of the 41 lambs and 71.4 per cent of these had harbored Pasteurella organisms in their throats during the nursing period. The dams may transmit the organisms to the lambs, which in turn may provide the organisms which produce mastitis in their dams.

Pasteurella organisms, pleuropneumonia-like organisms, and a viral agent were found to be associated with pneumonic lesions in lambs at the time of slaughter.

References

[1] Bosworth, T. J., and Lovell, R.: The Occurrence of Hemolytic Coccobacilli in the Nose of Normal Sheep and Cattle. J. Comp Path. & Therap., *54*, (1944): 168–171.

[2] Campbell, D., Campbell, R. S. F., and Dromery, L.: Pneumonia in Sheep Associated with Infection by a Pasteurella-like Organism. Vet. Rec., *61*, (1949): 64–65.

[3] Carter, G. R., and Schroder, J. D.: Pleuropneumonia-like Organisms Associated with Pneumonia in Swine. Canad. J. Comp. Med. & Vet. Sci., *19*, (1955): 219-220.

[4] Cordy, D. R., Adler, H. E., and Yamamoto, R.: A Pathogenic Pleuropneumonia-like Organism from Goats. Cornell Vet., *45*, (1955): 50–68.

[5] Creeche, G. T., and Gochenour, W. S.: Chronic Progressive Pneumonia of Sheep, with Particular Reference to Its Etiology and Transmission. J. Agric. Res., *52*, (1936): 667–679.

[6] Detweiler, R. S.: Hemorrhagic Septicemia in Sheep. Vet. Med., *26*, (1931): 123.

[7] Dungal, N.: Contagious Pneumonia in Sheep. J. Comp. Path. & Therap., *44*, (1931): 126–143.

[8] Gerloff, R. K., and Lackman, D. B.: Report of Wide Distribution of Psittacosis Antibody. Pub. Health Rep., *69*, (1954): 188–189.

[9] Greig, A. S.: The Isolation of Pleuropneumonia-like Organisms from the Respiratory Tract of Sheep. Canad. J. Comp. Med. & Vet. Sci., *19*, (1955): 265–271.

[10] Grumbles, L. C., Phillips, E., Boney, W. A., and Delaplane, J. P.: Cultural and Biochemical Characteristics of the Agent of Infectious Sinusitis of Turkeys and Chronic Respiratory Disease of Chickens. Southwest. Vet., *6*, (1953): 166–168.

[11] Hamdy, A. H.: Observations on Respiratory Disease Agents in Lambs. Ph.D. dissertation, Ohio State University, Columbus, 1958.

[12] Longley, E. O.: Contagious Caprine Pleuropneumonia. A Study of the Disease in Nigeria. Colonial Res. Publ. 7, H.M. Stationery Off., London, England, 1951.

[13] Macchiavello, A.: Cited by Meyer, K. F., and Eddie, B.: Psittacosis. In "Diagnostic Procedure for Virus and Rickettsial Diseases." 2nd ed. American Public Health Association Press, New York, N.Y. (1956): 420.

[14] Marsh, H.: The Role of Pasteurella in Sheep Diseases. J.A.V.M.A., *123*, (Sept., 1953): 205–208.

[15] McGowan, B., Moulton, J. E., and Sultz, G.: Pneumonia in California Lambs. J.A.V.M.A., *131*, (Oct., 1, 1957): 318–323.

[16] McKercher, D. G.: A Virus Possibly Related to Psittacosis-Lymphogranuloma Pneumonitis Group Causing a Pneumonia in Sheep. Science, *115*, (1952): 543–555.

[17] Morton, H. E., Smith, P. F., and Leberman, P. R.: Investigation of the Cultivation of Pleuropneumonia-like Organisms from Man. Am. J. Syph., *35*, (1951): 361–369.

[18] Newsom, I. E., and Cross, F.: An Outbreak of Hemorrhagic Septicemia in Sheep. J.A.V.M.A., *62*, (March, 1923): 759–762.

[19] Pounden, W. D., Bell, D. S., Edgington, B. H., and Thomas, D. L.: Disease Conditions Observed in Lambs at Slaughter. J.A.V.M.A., *128*, (March 15, 1956): 298–301.

XUM

[20] Saito, Y., Shimada, F., Nosu, S., Toda, M., and Imanishi, N.: Studies on the Non-bacterial and Infectious Pneumonia of Goats. I. Isolation and Morphological Findings of the Causative Agent. Kitasato Arch. Exptl. Med., *27*, (1954): 103–114.

[21] Salisbury, R. M.: Enzootic Pneumonia of Sheep in New Zealand. New Zealand Vet. J., *5*, (1957): 124–127.

[22] Spray, R. S.: Bacteriological Study of Pneumonia in Sheep. J. Infect. Dis., *33*, (1923): 97–112.

[23] Stamp, J. T., Watt, J. A. A., and Tomlison, J. R.: *Pasteurella Hemolytica* Septicemia of Lambs. J. Comp. Path. & Therap., *65*, (1955): 183–196.

Maternally Induced Protection of Young Lambs Against the *Epsilon* Toxin of *Clostridium Perfringens* Using Nonactivated Vaccine

LOUIS DS. SMITH, Ph.D., and TATS MATSUOKA, B.A.

Bozeman, Montana

THE PROTECTION of newborn lambs against enterotoxemia caused by *Clostridium perfringens* type D is attained most readily by passive immunization. This may be accomplished, in most cases, by the inoculation of each newborn lamb with antitoxin. Under field conditions, this method sometimes is not practical, and protection of the newborn lambs then can be achieved only by vaccinating the ewes and allowing the colostrum to transfer passive immunity to the lambs.

The immunity of the lamb would be expected to be a function of the immunity attained by the ewe. Consequently, it would be desirable to use a vaccine for the ewes which contained *epsilon* toxin-toxoid rather than *epsilon* prototoxin-toxoid, for the former has been shown to be more antigenic by Batty and Glenny [1] and Smith and Matsuoka.[5] However, not all commercially produced vaccines are prepared by trypsin activation.

Most workers who have investigated the problem of passively immunizing lambs by the active immunization of the ewes with nonactivated vaccine have given the ewes two injections during pregnancy. It is possible, however, that a satisfactory level of immunity could be attained with a single inoculation of nonactivated vaccine. Oxer [3] concluded that two inoculations were necessary, but the culture he used for the preparation of vaccine apparently contained little toxin and had not been precipitated with alum or another adjuvant. Boughton and Hardy [2] found that a single inoculation of a zinc chloride-precipitated vaccine into ewes caused an appreciable reduction in the incidence of enterotoxemia in their lambs. The vaccines that Oxer [3] and Boughton and Hardy [2] used were prepared without activation, and presumably consisted of prototoxin-toxoid.

It seemed possible to us that a single injection of a vaccine prepared from an alum-precipitated culture of high prototoxin content might induce sufficient immunity in the ewes to give protective levels of antitoxin in the lambs. While this was being tested, opportunity was taken to determine the duration of the passive immunity thus induced in the lambs. Other workers have demonstrated such passive immunity to last for five to six weeks, but it is not known whether it lasts longer.

MATERIALS AND METHODS

The animals used in this experiment were 25 grade Rambouillet and Columbia ewes, 3 years old. Some of these animals had been immunized with commercial enterotoxemia vaccine as young lambs (Smith and Marsh [4]). All of them had been handled together since that time and, presumably, all had the same opportunity of acquiring "natural" immunity by field exposure. Blood samples were taken from the ewes about one month before lambing was due to begin and they then were inoculated subcutaneously with 5 ml. of commercial alum-precipitated *Cl. perfringens* type D vaccine (Fringol *). This vaccine had 180 combining units per milliliter of prototoxin-toxoid and was prepared from a culture, a sample of which had approximately 41,000 mouse lethal doses (m.l.d.) of *epsilon* toxin per milliliter after activation with trypsin. The vaccine itself, however, was not treated with trypsin before formolization and precipitation with alum.

Blood samples were taken from each ewe on the day she lambed, and a sample of colostrum was taken. Blood samples were taken from the lambs when they were 2 weeks old and at approximately two-week intervals thereafter. The serums from the ewes, the colostrum, and the serums from the first sampling of the lambs were titrated for *epsilon* antitoxin content. The serums from the subsequent samplings of the lambs were tested only to see if they contained 0.5 American unit per milliliter or more. Serums were tested for antitoxin by the methods described by Smith and Matsuoka.[5] The results are expressed in American units (1.0 American unit is equal to approximately 0.1 international unit). Titrations were made at approximately two-fold differences. In calculating geometric means, the titer of each serum was taken as the midpoint of the range in which it fell.

Journal series paper No. 411 from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Agricultural Experiment Station, Montana State College, Bozeman.

This study was supported in part by a grant from Cutter Laboratories, Inc., Berkeley, Calif.

* Fringol is produced by Cutter Laboratories, Inc., Berkeley, Calif.

TABLE 1—Antitoxin in Serums of Lambs and in Serums and Colostrum of Ewes not Previously Immunized

Ewe (No.)	Lamb (No.)	Ewe serums before inoculation	Ewe serums after inoculation	Colostrum	Lamb serums
131	274	0.0	4.0*	15.0	1.0
63	292	0.0	0.5	4.0	0.5
	293				1.0
16	296	0.0	4.0	120.0	4.0
	297				7.0
26	299	3.0	29.0	29.0	15.0
	300				15.0
35	306	0.0	1.0	1.0	0.2
106	310	0.0	4.0	15.0	2.0
184	315	0.0	1.0	4.0	4.0
28	316	0.0	2.0	2.0	1.0
168	317	0.0	2.0	0.5	0.5
174	325	0.0	29.0	15.0	15.0
164	338	0.0	15.0	7.0	15.0
	339				4.0
8	346	1.5	7.0	15.0	7.0
	347				7.0
4	360	0.0	0.3	4.0	0.5
	361				0.5
91	366	0.0	15.0	59.0	2.0
25	425	0.8	15.0	29.0	7.0
	426				15.0
170	442	0.0	1.0	4.0	7.0
	443				0.5
Geometric means			5.21	12.0	4.02

* American units of *epsilon* antitoxin per milliliter of serum.

RESULTS AND DISCUSSION

The antitoxin content of the serum and colostrum of ewes given a single injection of vaccine one month before lambing is shown (table 1) along with the antitoxin content of the serums of their lambs. Only 3 of the 16 ewes had detectable antitoxin in their serums before vaccination. They responded fairly well to the vaccine, however, and the serums of all their lambs contained antitoxin at 2 weeks of age. The geometric mean of the antitoxin titers was 4.02 units per milliliter. Six of 24 (25%) of these lambs had not more than 0.5 unit of antitoxin per milliliter of serum, which probably is the lowest level that will provide protection against enterotoxemia in the field. It is lower than is desirable in lambs 2 weeks old, for the antitoxin level would be expected to drop subsequently, leaving some of the lambs insufficiently protected during a period of life when the incidence of enterotoxemia is high.

Similar information is given (table 2) for ewes which had been vaccinated twice—once as young lambs and again one month before lambing, at 3 years of age. The serums of 8 of these 9 ewes had demonstrable antitoxin before the prenatal vaccination. Apparently, the injection of vaccine given to them as young lambs had an appreciable effect on their immune status three years later. This is particularly surprising because 4 of them (No. Z-80, Z-83, Z-126, and Z-128) showed no immediate measurable responses to the vaccine given to them as

TABLE 2—Antitoxin in Serums of Lambs and in Serums and Colostrum from Ewes Previously Immunized

Ewe (No.)	Lamb (No.)	Ewe serums before prenatal inoculation	Ewe serums after prenatal inoculation	Colostrum	Lamb serums
80	294	0.40*	2.0	15.0	2.0
128	309	1.50	4.0	59.0	4.0
123	324	6.00	29.0	240.0	29.0
107	332	12.00	240.0	940.0	29.0
83	357	0.40	15.0	59.0	7.0
139	373	0.00	2.0	7.0	0.5
62	375	1.50	7.0	120.0	7.0
	376				7.0
31	422	0.38	15.0	7.0	4.0
126	449	6.00	59.0	470.0	7.0
	450				4.0
Geometric means			19.0	102.0	8.16

* American units of *epsilon* antitoxin per milliliter of serum.

TABLE 3—Duration of Immunity in Lambs from Immunized Ewes

Age (weeks)	Immune*/total	Immune (%)
2–3	24/25	96
4–5	24/25	96
6–7	21/25	84
8–9	15/19	79
10–11	21/25	84
12–13	15/18	83
15–19	10/25	40

* Lambs with 0.5 American unit or more *epsilon* antitoxin per milliliter of serum.

young lambs (Smith and Marsh [4]). The serum of only 1 of the 11 lambs (9%) belonging to these ewes vaccinated twice showed a level of antitoxin as low as 0.5 American unit, as compared with 25 per cent of the lambs from ewes that had been given only one prenatal inoculation.

With a few exceptions, the antitoxin levels of the lambs' serums were the same or only slightly lower than the levels of the serums of their dams. It is noteworthy, however, that lambs from ewes with the highest levels of antitoxin had only moderately more antitoxin than did the other lambs. Apparently, the amount of antitoxin that can be absorbed through the intestinal wall is limited and only a moderate level of passive immunity may be attained in this way.

It is apparent from these results that double vaccination of the ewes is desirable if newborn lambs are to be protected against enterotoxemia by a vaccine containing prototoxin-toxoid. It also is apparent that both inoculations need not be given during pregnancy, but that a considerable period may elapse between them. It seems likely that ewes could be given the first inoculation during their first year of life and thereafter be given one inoculation annually, a month or so before lambing, to protect their lambs against enterotoxemia.

The duration of this passive immunity could be followed in only 25 of the 35 lambs with which this experiment started, because of deaths caused by muscular dystrophy. Consequently, the lambs from both groups of ewes were considered together for this part of the study. From the results of antitoxin titrations at the 0.5-unit level (table 3), it is evident that the duration of this passively acquired immunity is long, for 80 per cent of the lambs showed at least minimum protective levels about three months after birth. Since the antitoxin levels resulting from the inoculation of lambs with heterologous antitoxin usually drop sharply in three to four weeks, it is apparent that passive immunity attained by maternal immunization lasts much longer. It also must be accepted, however, that an occasional ewe may respond poorly and her lamb may be insufficiently protected. This is the case when twin lambs share the colostrum of a single ewe or when one of a pair of twins gets almost all of the colostrum.

SUMMARY

A single injection of unactivated *Clostridium perfringens* vaccine into pregnant ewes provided presumably protective levels of antitoxin in 18 of 24 of their lambs. Two injections of unactivated vaccine, however, produced presumably protective levels in 10 of 11 of the lambs. The level of antitoxin in the colostrum and in the ewes' serums also was higher in the group given two injections, even though three years elapsed between the two injections. The majority of lambs from actively immunized ewes retained presumably protective levels of antitoxin for 12 weeks.

References

[1] Batty, I., and Glenny, A. T.: The Antigenic Efficiency of *Cl. Welchii Epsilon* Toxin and Toxoid after Treatment with Trypsin. Brit. J. Expti. Path., *29*, (1948): 110–126.

[2] Boughton, I. B., and Hardy, T. W.: Texas Agric. Exper. Sta. Rep. 153, (1940): 237.

[3] Oxer, D. T.: Transmission of Antitoxic Immunity from the Ewe, Vaccinated Against Enterotoxaemia, to the Lamb. Austral. Vet. J., *12*, (1936): 54–58.

[4] Smith, L. DS., and Marsh, H.: The Immunization of Young Lambs Against Enterotoxemia. Am. J. Vet. Res., *14*, (July, 1953): 408–410.

[5] Smith, L. DS., and Matsuoka, T.: A Comparison of *Clostridium Perfringens Epsilon* Toxin-Toxoid and Prototoxin-Toxoid in the Immunization of Young Lambs. Am. J. Vet. Res., *15*, (July, 1954): 361–363.

Septicemic Pasteurellosis in Lambs

ERNST L. BIBERSTEIN, D.V.M., Ph.D., and
PETER C. KENNEDY, D.V.M., Ph.D.

Davis, California

ALTHOUGH THE EXISTENCE and widespread occurrence of *Pasteurella hemolytica* has been known for over a generation,[5] only recently has any specific pathogenic activity, distinct from that of other members of the so-called "hemorrhagic septicemia" group of Pasteurella organisms, been assigned to that species. The role of this organism as a pathogen has, at times, been held in doubt.[8]

In the last few years, this situation has shown signs of change. In shipping fever pneumonia of cattle, *Past. hemolytica* has attracted increasing attention,[1,2] while three reports from different parts of Europe implicate this organism specifically in a disease of sheep, for which the time-honored designation of "hemorrhagic septicemia" appears apt.[11-13] Although this disease has been described in this country as a clinical and pathological entity, it has not been linked with any particular species of Pasteurella.[9] Moreover, "the available information. . . indicates that . . . classical hemorrhagic septicemia has been seen only rarely and, when observed, there has been doubt as to whether Pasteurella was the primary etiological agent."[7]

In view of the inconclusive and contradictory status that the disease occupies in the American literature, it is appropriate to describe our experiences in California relating to this condition, which is recognized with increasing frequency in the sheep raising areas of that state.

Six instances in which adequate data were available came to our attention in 1957. The histories were strikingly similar. The affected animals were feeder lambs in a satisfactory nutritional state. All or part of the flocks recently had been subjected to long shipment or shearing, or both. The time of highest local incidence, from May through September, was probably more a reflection on management practices than on seasonal predisposition. The mortality ranged from 1 to 2 per cent of the flock, but losses up to 5 per cent have been known to occur. The total incidence is more difficult to determine. The owner may state the number found dead and add that several times as many have been seen sick. Under the prevailing management conditions this is probably the best approximation obtainable. The signs of illness are vague and sometimes absent; the deaths of 2 or 3 animals often are the first indications of the disease. Descriptions by the owners, alerted by such losses, often reflect the observer's prejudices more than any objective change in the sheep. There are few specific diagnostic signs in the acute form.

BACTERIOLOGY

In nine of ten sufficiently documented outbreaks on record between 1954 and 1957, *Past. hemolytica* was isolated in pure culture from the affected organs of lambs reaching necropsy. In the one remaining instance, available information points to *Past. multocida*, although there is room for doubt. The study of Stamp *et al.*[11] emphasized the great variation in fermentation reactions during culturing of this species and invalidated many of the previous rigid standards. The criteria used by us are mainly the action on bovine erythrocytes in solid mediums, failure to produce indole, growth on McConkey's agar, and the usual morphological and cultural characteristics of this group of Pasteurella organisms. The hemolytic activity may vary considerably from strain to strain and appears to decline on continuous subculture. In the case of some strains, it is necessary to scrape away the colony to demonstrate the area of hemolysis, which coincides with that previously occupied by the colony. This fact, along with the emphasis placed by some texts on the fermentation of lactose, may account for some cases of *Past. hemolytica* infection having been reported in former

From the School of Veterinary Medicine, University of California, Davis.

Presented before the Section on Research, Ninety-Fifth Annual Meeting, American Veterinary Medical Association, Philadelphia, Pa., Aug. 18 to 21, 1958.

The authors thank Dr. Ottar Dybing, who translated the Norwegian reference material.

years as being due to Pasteurella spp. or even *Past. multocida.*

PATHOLOGY

We have recovered *Past. hemolytica* from a variety of lesions in sheep dead from diverse causes. In many of these, the pathogenic role of the organism has been obscure or in doubt. This report is concerned with the acute septicemic disease and its experimental counterpart. Necropsies were performed on 13 lambs; 10 had field cases and 3 had been experimentally infected.

Gross Findings.—Subcutaneous and intermuscular hemorrhages frequently were present, but often were small. Sometimes, subserosal hemorrhages were the striking lesion. They were particularly obvious in the epicardium, endocardium, and surrounding the mesenteric lymph nodes and the spleen.

The lungs were heavy and invariably failed to collapse completely when the thorax was opened. The severity of the pulmonary changes was variable but, in several, the lungs were slate blue and the air passages were filled with froth. Presumably as a result of the terminal dyspnea, the laryngeal mucosa was reddened and edematous. There were numerous dark red foci of hemorrhage scattered uniformly throughout the lung parenchyma. They were most easily visible through the serosa of the diaphragmatic lobes, and were 0.5 to 1.0 cm. in diameter.

Pale foci of the same size could be seen in the liver parenchyma but, like the lung lesions, were most obvious when viewed through the intact serosa. They were distributed uniformly throughout the liver and some were surrounded by a red halo.

Superficial abomasal ulceration frequently was present, but was thought to be a nonspecific change, as was the hyperemia seen in the colons of several of the lambs.

The spleens usually were only slightly enlarged, but the lymph nodes regularly were swollen and edematous. This lymph node enlargement was general but, frequently, regional nodes showed particular involvement, with hemorrhage and lymph stasis (fig. 1). The significance of this change was appreciated only toward the end of this series, so we are unable to comment on the pattern of their involvement. We have observed that, in both naturally and experimentally infected lambs, the nodes of a particular area may be selectively affected.

Microscopic Lesions.—Scattered uniformly throughout all portions of the lung were small bacterial colonies, associated with relatively minor inflammatory changes. Many appeared as reactionless capillary thrombi (fig. 2), but narrow zones of necro-

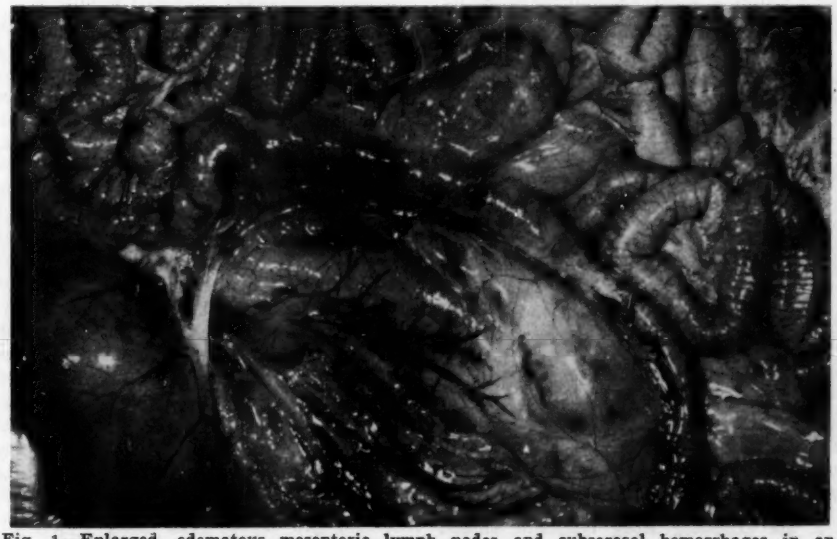


Fig. 1—Enlarged, edematous mesenteric lymph nodes and subserosal hemorrhages in an experimentally infected lamb.

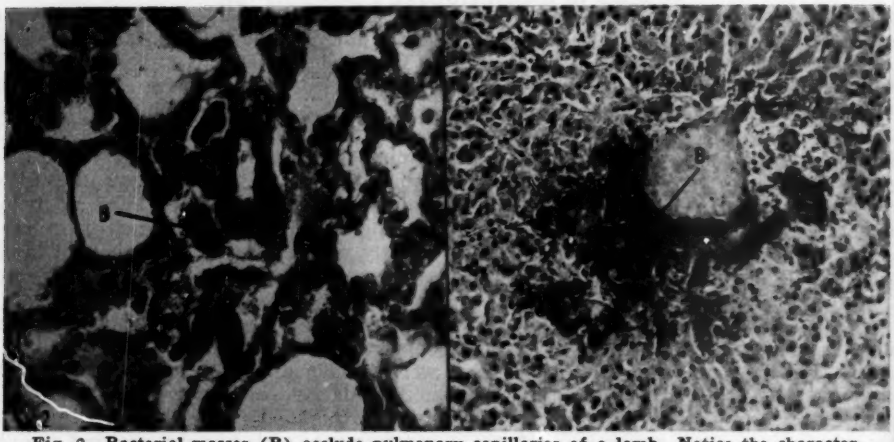


Fig. 2—Bacterial masses (B) occlude pulmonary capillaries of a lamb. Notice the characteristic lack of reaction. x 267.

Fig. 3—Bacterial colonization (B) in the liver of a lamb, with secondary portal vein thrombosis. x 267.

sis, hemorrhage, and edema surrounded others. Local neutrophilic infiltration appeared delayed and did not become confluent in any lambs in this series. In the most severely affected lungs, the process was spreading via the septal and subpleural lymphatics, where bacteria and thrombosis could be seen. The hepatic lesions were similar to the lung lesions and always appeared to be at the same stage of development. The bacterial colonies most frequently were located at the periphery of the lobules (fig. 3). The distribution within the liver was entirely uniform. As in the lung, many bacterial colonies had not evoked any inflammatory reaction. If a zone of necrosis surrounded the bacteria, it was rarely thicker than one hepatic cell layer. Hemorrhage and edema were less obvious than in the lung, but the neutrophilic infiltration was similar and usually modest.

Bacterial colonies regularly were seen in the adrenal cortex and in the spleen. Certain organs showed at least a relative resistance to colonization. Only one bacterial colony was found in a kidney and, except for the lymph nodes, which deserve special mention, sites of bacterial multiplication were seen infrequently in other organs.

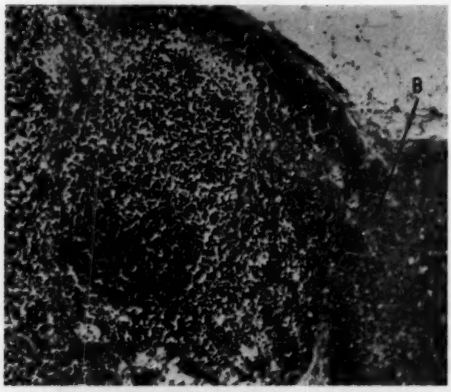


Fig. 4—Cervical lymph node in a lamb with a natural case of pasteurellosis. The entire subcapsular sinus is filled with bacteria (B) and masses of them are extending along lymphatic sinuses towards the medulla. x 267.

Remarkable bacterial multiplication was observed in the lymph nodes of 4 lambs. In 2 with natural infections, the cervical nodes were involved; in 2 infected by intravenous inoculation, the mesenteric nodes were affected. In these nodes, bacteria filled the subcapsular sinus and extended toward the medullary areas (fig. 4). The numbers of bacteria seen in these nodes far exceeded those seen in other organs. As in other sites, the numbers of organisms contrasted sharply with the degree of inflammation. All nodes were edematous and congested but, in the experimentally infected lamb that died after 18 hours, neutrophils were rare. This failure of neutrophilic response was, at least in part, a reflection on the

acuteness of the disease because, both in lambs with field cases and in the experimentally infected lamb which survived for 36 hours, relatively large numbers of neutrophils were associated with the masses of bacteria.

PATHOGENICITY TRIALS

Lambs involved in the last outbreak that came to our attention in 1957 recently had been shipped from Idaho and Montana and showed such unusual and severe lesions that tularemia also was considered. Pooled suspensions of liver, lung, and spleen were injected in 0.5-ml. amounts into guinea pigs and, in addition to the usual mediums, hemoglobin-cystine-glucose agar was inoculated. The only organism recovered on mediums was *Past. hemolytica*, but 2 of the 4 guinea pigs died within 24 and 48 hours, respectively. This development, unexpected since all reports but one [4] agree on the nonpathogenicity of *Past. hemolytica* for guinea pigs, suggested further trials.

MATERIALS AND METHODS

Guinea pigs were inoculated with bacterial suspensions or broth cultures by the intraperitoneal, intracardial, and intranasal routes.

Mice and hamsters were given intraperitoneal injections.

Chickens were injected intravenously.

Infection of lambs was attempted by subcutaneous, intravenous, and intranasal routes. A DeVilbiss atomizer was used to introduce the inoculum into the nasal passages and pharynx.

Inocula used in connection with pathogenicity tests were plated out in tryptose agar (Difco). It was found that a bacterial suspension of an optical density corresponding to the MacFarland nephelometer tube No. 1 contained about 2×10^8 viable cells, while a 24-hour broth culture tube contained approximately 10^9 organisms per milliliter. Estimation of dosages was based on these findings.

The strain used in all tests was the one isolated from the outbreak referred to in the preceding section.

RESULTS

Guinea Pigs.—Pure cultures of the field strain were shown to be capable of killing guinea pigs of all ages by intraperitoneal and intracardial inoculation. Fatal infections were induced with dosages of 20 to 500 million organisms. An occasional guinea pig appeared resistant. On one occasion, an injected guinea pig aborted 2 fetuses from which *Past. hemolytica* was isolated, while showing no other disturbances. Death in infected animals occurred usually between 18 and 48 hours. Illness often was manifested within four to six hours, as shown by a ruffled hair coat, reluctance to move, and a hunched-up appearance. Occasionally, body temperatures on the first day after infection were as high as 107 F. This temperature rise must be a transient phenomenon, as it could not be demonstrated consistently. The temperature of visibly sick animals commonly was normal or subnormal. In the latter case, they invariably died.

At necropsy, the guinea pigs given intraperitoneal injections had an unusual fibrinous peritonitis and a greatly reddened, injected omentum. The lobes of the liver were joined by sheets of fibrin and, when the diaphragm was separated from the parietal surface of the liver, fibrinous strands were seen to connect the two organs (fig. 5). A large quantity of a thin, turbid fluid filled the peritoneal cavity. When this fluid was streaked on a slide and stained with Churchman's stain [3] or Klieneberger's modification of Giemsa's stain,[6] it revealed prodigious numbers of distinctly encapsulated pleomorphic rods, many of which showed bipolarity (fig. 6). Few inflammatory cells were present, and the extent of phagocytosis was not impressive. Except for pulmonary congestion, no thoracic lesions were seen.

Intracardial injections of numbers of organisms comparable to those used in intraperitoneal inoculations resulted in death in 24 hours. Few gross lesions were observed.

Intranasal instillation of up to 1 ml. of a suspension containing an estimated 10^9 organisms was without any noticeable effect.

Mice.—The doses required to cause death in 15- to 25-Gm. mice were about the same as those killing 300- to 500-Gm. guinea pigs. The course of illness was much the same in the two species, except that lesions were minimal in mice. Mouse pathogenicity was maintained in the test strain for over six months, during which time it was cultured on blood agar and transferred weekly. Intraperitoneal injection sometimes produced uneasiness and discomfort in 30 minutes. It was suspected that the disease in mice might be purely an intoxication due to the cellular components of the organisms contained in the inoculum, but identical doses of bacteria inactivated by 0.3 per cent

XUM

Fig. 5—Peritonitis in a guinea pig after intraperitoneal inoculation. Fibrinous strands of exudate and bacteria extend between adjacent organs.

formalin or heating at 56 C. for 30 minutes proved completely innocuous for mice. Filtrates of broth cultures also were innocuous. It therefore is clear that multiplication of the organisms must take place in susceptible mice, a fact also suggested by the presence of the bacteria in the heart blood at the time of death.

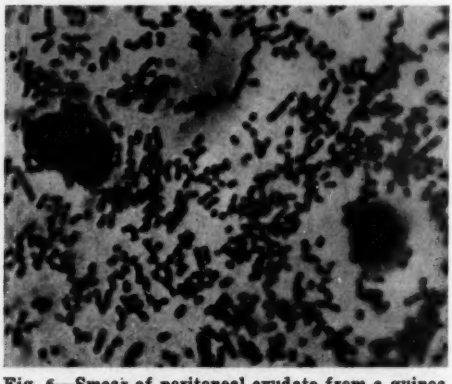


Fig. 6—Smear of peritoneal exudate from a guinea pig inoculated with 0.5 ml. of broth culture intraperitoneally. Churchman-Emelianoff stain; x 1,300.

Young hamsters proved refractory to doses lethal for mice and guinea pigs. Six-week-old chickens, injected intravenously with 5×10^8 organisms, failed to react in any observable manner. Rabbits injected by the same route also showed no evidence of susceptibility.

The pathogenicity experiments on lambs are summarized (table 1). All animals given parenteral injections of organisms showed reactions varying from a mild thermal response, which subsided after two or three days, to peracute or chronic disease. The latter forms, which duplicate the disease in the field, were seen only after the intravenous injection of large numbers of bacteria. Intranasal atomization of bacterial suspensions, even when accompanied and followed by injections of corticosteroids (Cortone*), failed to elicit any effect.

Bacteriologically, several interesting observations were made. One was the appearance of a variant form of *Past. hemolytica* in cultures from the lung, liver, and spleen of 3 lambs with fatal cases. The colonies first attracted attention because of their apparently stronger hemolytic action on beef blood agar plates, as compared to the standard type. This impression, however, may be due to less depth and greater transparency of the variant type of colony. Subsequent studies, to be reported separately, revealed additional differences in crystal violet uptake, mouse pathogenicity, and antigenic constitution. Biochemically, the two types appear identical. Although mice and guinea pigs apparently are less susceptible to fatal infection with the variant, both the acute and chronic disease was reproduced in lambs by intravenous injection of this organism. No reversion to the more common type was observed in such infections.

In lambs with arthritis, *Past. hemolytica* could be recovered from aspirated purulent joint fluid up to a month after infection. One affected lamb (347) which failed to improve was killed after ten weeks. Attempts to culture the organism from the joints most severely involved were unsuccessful at this time, despite the use of heavy inocula.

Necropsy findings in lambs dead from

* Cortone is produced by Merck Sharp and Dohme, Rahway, N.J.

acute infections were indistinguishable from those found in lambs with the spontaneous condition and, therefore, need not be described separately. Additional lesions encountered in lambs with the less acute and chronic experimental cases included arthritis, pericarditis, meningitis, and bacterial endocarditis involving the tricuspid valve. With the exception of the latter, all these lesions also have been reported or seen by us in field cases of *Past. hemolytica* septicemia.

DISCUSSION

Field observations and transmission experiments have satisfied us that a disease, identical in almost every detail with that described by Stamp *et al.*,[11] in Scotland, and Woxholtt *et al.*,[13] in Norway, does exist in this country and is the source of considerable loss to lamb feeders. It is difficult to relate the condition to the various descriptions of pasteurellosis, shipping fever, enzootic pneumonia, and other diseases. The pneumonic involvement generally associated with pasteurellosis apparently is slight in this disease and, when present, is of questionable specificity. In this connection, it is of special interest that, in 2 experimental lambs which survived for five and 12 days, respectively, no lung lesions qualitatively different from those found in lambs with acute cases were observed. It is possible that the acute pneumonia associated with shipping fever may develop through an entirely different mechanism.

Aside from the quoted reports from abroad, no published evidence has been presented to assign a specific pathogenic role to *Past. hemolytica* as the cause of septicemic pasteurellosis in lambs. Newsom,[9] who described this infection from a clinical and gross pathological viewpoint, implied that both *Past. multocida* and *Past. hemolytica* have roles of equal prominence in its cause. He also suggested that *Past. multocida* is the predominant cause of the disease in feeder lambs, while *Past. hemolytica* is found more often in breeding flocks and nursing lambs. Since neither of these statements applies to the situation in California (or, apparently, in Scotland and Norway), perhaps an explanation for this discrepancy may be found in the relative abundance of the two species in their supposedly natural reservoir, *i.e.*, the upper respiratory tracts of healthy sheep. An investigation of this sort is underway and thus far has shown that nasal swabs from 35 apparently healthy sheep yielded 31 strains of *Past. hemolytica* and 11 of *Past. multocida*. The identification was based on cultural and morphological characteristics. This numerical relationship may in part explain the preponderance of *Past. hemolytica* as the cause of septicemia in this area. It would be essential to know whether they occur with equal frequency in normal sheep in regions

TABLE 1—Summary of the Experiments with Pasteurella Infections in Lambs

Lamb (No.)	Inoculum	Dose	Route	Results
507	Broth culture filtrate (Seitz EK)	10 ml.	i.v.	None
527	Slant washings	2×10^9	s.c.	Transient fever
533	Slant washings	2×10^9	i.v.	Death in 18 hr.
531	Guinea pig liver	1 Gm.	i.v.	Transient fever
535	Guinea pig liver	1 Gm.	s.c.	Transient fever
543	Mouse liver	1 Gm.	i.v.	Transient fever
532	Mouse liver	1 Gm.	s.c.	Local inflammation, transient fever
Ram	Slant washings	2×10^9	i.v.	Death in 18 hr.
Ewe	Lamb liver (533)	1 Gm.	i.v.	Death in 5 days
344	Broth culture	5×10^8	i.v.	Death in 36 hr.
345	Broth culture	5×10^8	i.v.	Polyarthritis
346	Broth culture	5×10^8	i.v.	Death in 12 days
347	Broth culture	5×10^8	i.v.	Polyarthritis
348	Broth culture	5×10^8	i.v.	Transient temperature rise
349	Broth culture	5×10^8	i.v.	Arthritis
231	Slant washings	10^9	i.v.	Polyarthritis, transient fever
235	Slant washings	10^9	i.n. + cort.* i.m.	None
236	Slant washings	10^9	i.n. + cort.* i.m.	None

* Cort.= corticosteroids in 30-mg. doses.
i.v.= intravenous; s.c.= subcutaneous; i.n.= intranasal; i.m.= intramuscular.

XUM

where both organisms are equally prevalent as pathogens.

While we, as well as Scottish and Norwegian investigators, have succeeded in duplicating experimentally the clinical and pathological features of septicemic pasteurellosis in lambs, no claim can be made that the infectious process has been clarified by these demonstrations of pathogenicity. The large doses and the parenteral route of inoculation, which have been found essential for setting up the experimental disease, have no close counterpart in nature. It generally is postulated, therefore, that a certain devitalizing experience is necessary to render the tissues receptive to the entrance and multiplication of the parasite. The conditions under which the disease is encountered lend support to such assumptions. It also has been suggested that there may be differences in virulence between strains.[3] Our identification of a variant type, first in tissues of experimentally infected lambs and subsequently from the nasal mucus of normal sheep, may have some bearing on this point. Although, when given in large intravenous doses, this variant strain induced the same disease in lambs as the standard type isolated originally, it was consistently of lower pathogenicity for mice and has failed to produce death in guinea pigs. Similarly, of six strains recovered from sources other than animals with septicemia, only one has proved pathogenic for guinea pigs. We subscribe to the view, expressed by Florent and Godbille,[4] that the pathogenicity for experimental animals is dependent largely on the strain used.

An observation pertinent to the question of strain differences concerns the comparative behavior of the original strain and the nasal isolates on subculture. The former, now on artificial mediums for eight months, has remained smooth and never has been found to give rise to dissociants in culture during that period, while the strains obtained from normal sheep have shown strong dissociative tendencies. The colonial form of most strains originally was intermediate between the smooth and variant types. Only through many serial subinoculations from single colonies have pure smooth and variant lines been gradually established from some.

The strikingly different behavior of the one indisputably pathogenic strain from the apparently commensal types suggests an additional factor in the genesis of septicemic pasteurellosis, which we intend to explore further.

SUMMARY

Pasteurella hemolytica septicemia, as described by Stamp *et al.*[11] in Scotland, exists in identical form in California and has been reproduced in lambs by the intravenous inoculation of pure cultures recovered from lambs with field infections.

The predominant gross lesions were serosal hemorrhages and congestion and edema of the lungs and lymph nodes. Focal lesions in the lung appeared to be small areas of hemorrhage; in the liver, these areas were pale. Microscopically, the presence of distinct bacterial colonies in the lungs, liver, spleen, and adrenal cortex was a constant and characteristic finding. The striking feature was that no well-developed inflammatory reaction was associated with them, even in lambs surviving for more than 36 hours.

Experimental lambs surviving beyond the first day developed such complications as arthritis, pericarditis, and meningitis. No pneumonia comparable to the field condition associated with shipping fever has been seen in experimentally infected lambs.

The finding of a colonial variant and the demonstration of cultural differences between a pathogenic strain and isolates from normal sheep point to the possibility that characteristics of the parasite, as well as host factors, are apt to be involved in the pathogenesis of septicemic pasteurellosis.

References

[1] Carter, G. R.: Observations on the Pathology and Bacteriology of Shipping Fever in Canada. Canad. J. Comp. Med., *18*, (1954): 359–364.

[2] Carter, G. R., and McSherry, B. J.: Further Observations on Shipping Fever in Canada. Canad. J. Comp. Med., *19*, (1955): 177–181.

[3] Churchman, J. W., and Emelianoff, N. V.: A Study of the Bacterial Capsule by New Methods. J. Exptl. Med., *57*, (1933): 485–510.

[4] Florent, A., and Godbille, M.: Les pasteurellas (coccobacilles) hémolytiques et leur importance dans la pathologie animale en Belgique. Leur sensibilité à la pénicilline. Ann. méd. vét., *94*, (1950): 337–361.

[5] Jones, F. A.: A Study of *Bacillus Bovisepticus*. J. Exptl. Med., *34*, (1921): 561–577.

[6] Klieneberger-Nobel, E.: Methods for the Study of the Cytology of Bacteria and Pleuropneumonia-like Organisms. Quart. J. Micros. Sci., *91*, (1950): 340–347.

[7] Marsh, H.: The Role of Pasteurella in Sheep Diseases. J.A.V.M.A., *123*, (Sept., 1953): 205–208.

[8] Montgomerie, R. F., Bosworth, T. J., and Glover, R. E.: Enzootic Pneumonia of Sheep. J. Comp. Path., *51*, (1938): 87–107.

[9] Newsom, I. E.: Sheep Diseases. Williams and Wilkins Co., Baltimore, Md., 1952.

[10] Smith, G. R.: Discussion on A. J. Stevens' "Respiratory Diseases of Sheep" (Proc. 75th Ann. Cong. Brit.

Vet. A., Cambridge, Aug. 25–31, 1957). Vet. Rec., *69*, (1957): 1249–1257.

[11] Stamp, J. T., Watt, J. A. A., and Tomlinson, J. R.: *Pasteurella Hemolytica* Septicemia in Lambs. J. Comp. Path., *65*, (1955): 183–196.

[12] Stricker, F., and Grunert, Z.: *Pasteurella hemolytica* ako pôvodka septikemického ochorenia oviec. Vet. Cas., *5*, (1956): 435–440 [abstr. in Vet. Bull., *27*, (1957): 449].

[13] Woxholtt, C., Naerland, G., and Hoff, H.: *Pasteurella Hemolytica* Infeksjon hos sau. Nord. Vet.-med., *4*, (1952): 433–450.

Experimental Strongyloidiasis in Sheep and Goats
I. Single Infections

JAMES H. TURNER, Ph.D.

Beltsville, Maryland

To EVALUATE further the pathogenic potentialities of *Strongyloides papillosus* (Wedl, 1856), the intestinal threadworm of domestic ruminants, an experiment was conducted to determine the pathogenicity of uncomplicated infections of this parasite in lambs and kids. The purpose of this paper is to present a more complete account of the results of this experiment than that reported previously.[20]

Cattle, sheep, goats, and certain wild ruminants are the natural hosts of *S. papillosus*, but rabbits and man can be infected experimentally. However, this parasite was not regarded as a dangerous pathogen until recently, primarily because few experimental studies have been made of its effects on the natural hosts.

Roberts [15] observed a debilitating effect of this parasite on a naturally infected calf, but Orlov,[14] on the basis of an inconclusive experiment involving small numbers of larvae, stated that *S. papillosus* had little pathogenicity for sheep. Woodhouse [24] experimentally infected 1 lamb and 1 steer, and demonstrated the pathogenicity of *S. papillosus* in domestic ruminants. Recently, Garkavi [9] reported that 4 of 9 lambs died after experimental infection with 200,000 or more *S. papillosus* larvae.

So far as the author can ascertain, these reports [9, 14, 24] are the only ones in the literature dealing with experimental infections of this parasite in sheep. No experimental studies on goats have been reported except the cross-transmission experiment of Vegors and Porter.[23] These authors observed no pathogenic effects in any of their experimental animals. Vegors [22] first investigated experimentally the pathogenicity of strongyloidiasis in calves, and reported acute effects resulting from uncomplicated infections.

Clinical signs observed by one or more of these investigators included diarrhea, progressive emaciation, anorexia, cachexia, and polyuria. To the author's knowledge, anemia associated with strongyloidiasis in any ruminant was first reported by himself,[20] and only once [9] afterward. Deaths of calves from experimental infections of *S. papillosus* were reported by Vegors,[22] and the death of a ram by Woodhouse.[21] Reported deaths of ruminants ascribed to a natural infection of this parasite are few; the death of a calf was reported by Woodhouse,[24] and the deaths of 3 lambs by Turner and Wilson.[21]

EXPERIMENTAL PROCEDURES

Lambs and kids used in these experiments were removed from their mothers at birth. They were taken immediately to a parasitologically sterile isolation building and placed in clean wire cages. Before weaning, the animals were fed a formula of milk and vitamins which was supplemented by chopped alfalfa hay and mixed grain at 4 weeks of age. The animals were weaned when 8 weeks old, and the hay and grain ration was fed thereafter. Infected and control animals were fed the same quantity of feed daily.

All animals were maintained from birth in wire cages which were cleaned daily. Various other routine precautions were taken to prevent extraneous infection. Sterilized coveralls and overshoes were worn while working in the isolation quarters, and a foot bath of lye solution was placed inside the entrance to the building to disinfect the overshoes. The cages and floor of the building were disinfected semiweekly. Therefore, all animals used in these experiments were free of helminth infections at the time of exposure to *S. papillosus*. However, this method for rearing helminth-free lambs and kids was not completely effective in preventing infection with coccidia, as approximately one third of the animals acquired subclinical infections of one or more species of Eimeria about two to three months after birth.

Infective larvae were obtained from standard charcoal cultures of feces from lambs infected previously with *S. papillosus*. They were collected by washing them from the sides of the containers on the day the animals were infected, approximately five days after preparation of the cultures. The larvae were counted by a routine dilution technique and separated into doses for administration to individual animals.

Of the 12 Shropshire lambs and 3 Toggenburg kids used in this work, 8 lambs and 2 kids were infected with *S. papillosus* by single cutaneous applications of infective larvae (table 1). These animals were restrained on their backs, with legs spread and tied; the wool was clipped from the inguinal regions, and infective larvae in water were applied to the skin of the clipped region by pipette. The time required for larval penetration varied, but usually was less than 45 minutes. The animals were returned to their cages when

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

This paper is part of a thesis submitted to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the Ph.D. degree.

The author acknowledges the assistance of Messrs. G. I. Wilson and W. N. Smith in various phases of this work.

TABLE 1—Larval Exposures and Number of Adult Strongyloides Papillosus Recovered at Necropsy

Animal (No.)	Age and weight when infected (days)	(lb.)	No. larvae put on skin (thousands)	Time of necropsy (days post-infection)	Worms recovered: Abomasum	Small intestine	Total	Percentage of larvae given
L521 †	57	19.0	25	44	0	2,225	2,225	8.9
L512	59	20.5	50	56	0	10,000	10,000	20.0
L522	57	22.0	75	56	0	22,500	22,500	30.0
L513 *	58	24.0	100	41	20	18,300	18,320	18.3
L538 *	75	22.5	200	18	350	135,700	136,050	68.0
L527 *	78	25.0	300	16	0	277,900	277,900	92.0
L529 *	77	27.5	500	13	340	119,800	120,140	24.0
L514 *	92	32.0	1,000	13	0	310,600	310,600	31.6
K284	61	17.0	100	83	0	6,970	6,970	6.9
K275 *	55	22.0	300	21	0	75,950	75,950	25.1

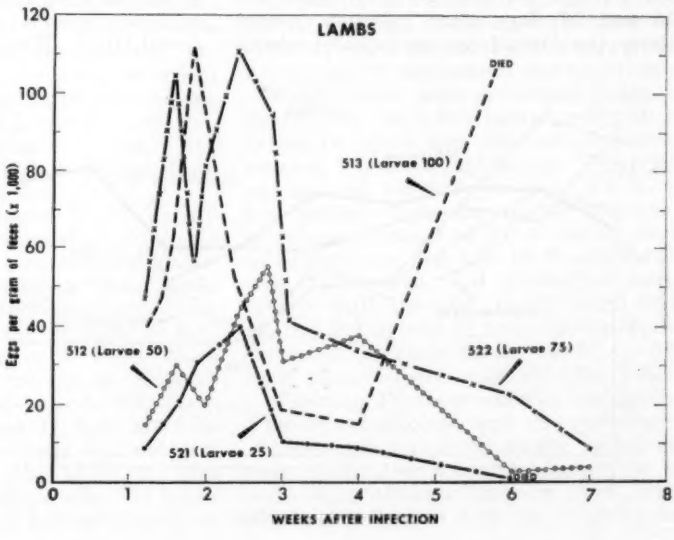
* Died of strongyloidiasis; † died of Strongyloides infection complimented by coccidiosis.
L = lamb; K = kid.

the water in which the larvae were suspended had dried on the skin and larval penetration was essentially complete. The 4 helminth-free Shropshire lambs and 1 Toggenburg kid served as uninfected controls. All lambs and kids were given the same rations daily.

Daily observations were made for the appearance and severity of clinical signs of infection in the lambs and kids. Fecal egg counts were made daily for the first three weeks after infection and semiweekly thereafter. Rectal temperatures of the lambs only were ascertained daily at 3:00 p.m. for the first 26 days after infection, the period when the effects of the infection were most acute.

Packed erythrocyte volume (hematocrit) and hemoglobin determinations were made weekly on all animals. Blood samples were drawn by puncturing the jugular vein with a No. 15 needle. The blood was collected in 10-ml. vials containing a 2:3 mixture of potassium and ammonium oxalate as the anticoagulant. Blood hemoglobin levels were determined by the cyanmethemoglobin method described by Turner.[39] The packed erythrocyte volume was determined by centrifugation of oxalated blood in standard Wintrobe tubes at 2,500 r.p.m. for 90 minutes. The infected and control groups of animals were weighed weekly in separate weighing cages.

Parasites were recovered from the animals at necropsy by the following procedure: Immediately after opening the abdomen, the small intestine was tied at the pyloric and ileocecal junctions and removed. The entire organ was slit longitudinally, and the contents washed out. It then was stripped through the fingers several times to free all worms lodged in the intervillous spaces or in the mucosa, and washed again after each stripping. The solid material from these washings, including the worms, was concentrated by sedimentation and preserved in 4 per cent formalin. Total worm counts were derived from the average number of worms present in small, duplicate aliquots taken from mate-



Graph 1—Number of Strongyloides papillosus eggs per gram of feces of lambs exposed to single applications of 100,000 or fewer larvae (larvae in thousands).

XUM

rial collected from each of the experimentally infected animals. The remaining viscera were examined for gross lesions, and small pieces of affected organs were excised, sectioned, and stained by routine methods for histological study.

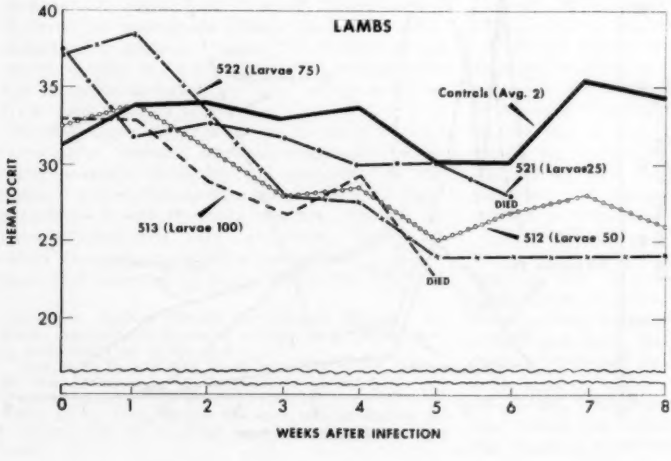
RESULTS

Clinical Findings—Lambs.—Eggs first appeared on the ninth day after infection (graph 1) in the feces of the lambs exposed to single applications of 25,000 to 100,000 infective larvae. Thereafter, the number of eggs in the feces of the infected lambs increased rapidly, usually reaching a peak of from 40,000 to more than 100,000 eggs per gram during the second or third week after infection. In lamb 513, there was also an abrupt increase in the number of eggs two weeks before its death, whereas in the 3 surviving lambs exposed to fewer than 100,000 larvae, the fecal egg counts gradually decreased after a maximum had been attained, so that by the sixth or seventh week after infection they had decreased to less than 500 eggs per gram.

Eggs also appeared first on the ninth day after infection (graph 4A) in the feces of the lambs exposed to 200,000 or more larvae except in the case of lamb 514, from which no fecal sample was obtainable before its death, 13 days after infection. The maximum fecal egg counts of lambs from both groups were related directly to the quantities of larvae used to infect the lambs, except those for lambs 527 and 529. In these 2 lambs, which died 16 and 13 days after infection, respectively, the peak of egg production probably had not been attained at the time of death, as an upward trend prevailed in the egg counts of these lambs until death occurred.

A decrease in hematocrit and hemoglobin levels occurred in all the infected lambs during the experiment, beginning during the first or second week after infection. The average hematocrit level of lambs exposed to 100,000 or less larvae (graph 2) decreased from 34.4 to 26.0 per cent during the experimental period. The greatest decline, 37.0 to 24.0 per cent, occurred in lamb 522, which was exposed to 75,000 larvae. The average decline in hemoglobin level was 2.6 Gm./100 ml. of blood, or from 11.4 to 8.8 Gm.; the greatest decrease, from 12.0 to 8.4 Gm., also occurred in lamb 522. The hematocrit and hemoglobin levels of the control lambs were slightly higher at the end of the experiment than at the beginning.

The 4 lambs exposed to 200,000 or more larvae died in less than three weeks after infection, and their average hematocrit values decreased from 33.7 to 24.5 per cent, as compared with a slight average decrease of 36.0 to 30.0 per cent in the controls (graph 4C); their average hemoglobin levels decreased 3.0 Gm., whereas those of the controls decreased only 1.8 Gm. The most significant hematological change occurred in lamb 538, which was infected with 200,000 larvae. Its hematocrit level declined from 34.0 to 18.0 per cent, and its hemoglobin level from 11.4 to 6.5 Gm. It is evident from these data that a slight to moderate anemia developed in these infected lambs. Hematological data for lamb



Graph 2—Changes in the hematocrit levels of lambs exposed to single applications of 100,000 or fewer larvae, as compared with those of the uninfected controls (larvae in thousands)

514 are not included in these averages, as no blood was obtained from this lamb at necropsy.

A significant loss of weight occurred in only 2 lambs of the 4 infected with 100,000 or fewer larvae (graph 3). Lamb 521, given 25,000 larvae, lost 2.0 lb. between the third and fourth weeks after infection, but gained 4.0 lb. before its death, seven weeks after infection. Lamb 513, given 100,000 larvae, progressively lost weight from the second week of the experiment until its death, 41 days after infection. At this time, the animal weighed 6.0 lb. less than its original weight of 24.0 lb. Lambs 512 and 522, given 50,000 and 75,000 larvae, respectively, gained 14.5 and 10.0 lb., respectively (av., 12.25 lb.), whereas 2 control lambs gained 15.0 and 19.0 lb., respectively (av., 17.0 lb.), during the same period. As early as one week after infection, a slight loss in weight occurred in lambs 527, 529, and 514, which were exposed to 300,000 or more larvae (graph 4B). All the lambs infected with 200,000 or more larvae weighed 2.5 to 6.0 lb. less at the time of death than at the start of the experiment.

The body temperatures of both infected and control lambs remained normal (100–104 F.) throughout the experiment.

Clinical signs common to the acutely affected lambs were inappetence, weakness, lethargy, difficult respiration, abnormal thirst, polyuria, and abnormal stools. Polyuria was especially noticeable in lambs 527, 513, and 514 about a week before death. During this period, the water troughs in their isolation pens had to be filled several times daily, whereas one full trough daily was ample for the controls. The consistency of the feces of the affected lambs several days before death varied from formed to fluid.

Of the 8 infected lambs, the 5 exposed to 100,000 or more larvae died within 13 to 41 days after infection from effects attributable to strongyloidiasis. The 2 lambs given 500,000 and 1,000,000 larvae, respectively, died on the thirteenth day; otherwise, the rapidity with which death occurred was directly related to the number of larvae applied. Lamb 521, given 25,000 larvae, also died 44 days after infection, but strongyloidiasis was not considered the primary cause of death. This animal acquired a massive coccidial infection of *Eimeria ninae-kohl-yakimovi*, despite precautions taken to prevent extraneous infections. This infection was first diagnosed 28 days after the lamb had been exposed to *S. papillosus*. Lambs 512 and 522, exposed to 50,000 and 75,000 larvae, respectively, survived the infection.

Clinical Findings—Kids.—The clinical effects of strongyloidiasis were similar in the lambs and kids. Eggs first appeared in the feces of the kids nine days after infection, and maximum egg counts of more than 100,000 eggs per gram were observed early in the third week after infection. Kid 275 died 21 days after infection, at which time 6,120 eggs per gram were recorded. Kid 284 survived to the end of the experiment, and its fecal egg count gradually decreased to 1,980 eggs per gram by the eighty-second day after infection.

Anemia developed in both of the infected kids. During the three weeks after infection, the hematocrit level of kid 275 decreased from 31 to 16 per cent, and its hemoglobin level from 10.0 to 5.4 Gm./100 ml. of blood. The anemia probably would have become more severe in this kid had not death intervened. The hematocrit level of kid 284 declined 27 to 8 per cent, and its hemoglobin level fell from 9.4 Gm. to the minimum level of 3.5 Gm. by the seventh week after infection. By the eleventh week after infection, this kid had made a partial recovery from its anemia, at which time its hematocrit reading was 21 per cent and its hemoglobin level was 7.8 Gm./100 ml. of blood. The hematocrit and hemoglobin levels of the control kid were within normal limits throughout the experiment.

Weight gains were subnormal in the infected kids. Kid 275 gained only 3.0 lb. before its death and was listless and depressed, but continued to eat. The terminal gain of kid 284 was only 6.5 lb. It had gained weight slowly and attained a maximum increase of 8.0 lb. seven weeks after infection, but lost 4.0 lb. during the next two weeks, and thereafter gained slowly until the experiment ended. The control kid gained 19.0 lb. during the 12 weeks of the experiment, which was 12.5 more than the surviving kid (kid 284).

Necropsy Findings—Lambs and Kids.—The organs affected and the pathological changes involved were similar in all animals dying from strongyloidiasis. The rumens and abomasa usually were partly filled with food at necropsy. Ascites and

XUM

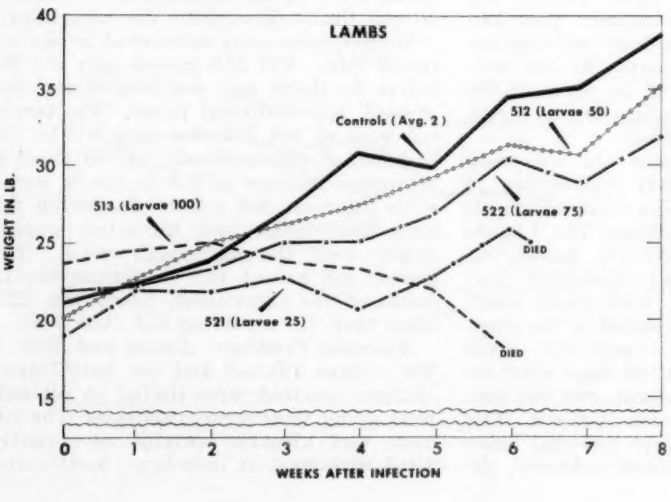
hydrothorax were seen commonly, as well as liquid material filling the small intestine and, sometimes, the large intestine. The cecal contents were semisolid or liquid. A typical catarrhal enteritis, accompanied by considerable exudate, occurred along the length of the small intestine. There was marked erosion of the mucosal epithelium of the duodenum and, to some extent, of the jejunum. Some thickening of the wall, inflammation, hyperemia, and edema sometimes were present in the first few feet of the intestine. In histological sections, the mucosal epithelium of the duodenum appeared almost entirely destroyed, often almost to the muscularis mucosa. Leukocytic infiltration and edema also were observed. Sections of nematodes were seen deep in the intestinal glands, but not in the submucosa.

Lung lesions were found only in lamb 514, which died only 13 days after infection. There was congestion in the anterior portion of the diaphragmatic lobes, and some emphysema in the basal lobe of the right lung. A pneumonitis also was present, probably secondary to the mechanical irritation and damage caused by the migrating larvae. Histological preparations of the lung tissue showed massive hemorrhage with serous exudate and extravasated blood within the tissue and alveoli. Apparently, any damage to the lungs caused by prior migration of larvae through them had been repaired in the majority of animals by the time they died.

The abdomen of lamb 521, whose death was complicated by concurrent coccidiosis, was greatly distended with gas, and a moderate amount of fluid was found in the pleural cavity. The rumen and abomasum were half filled with food and extremely swollen. There was little chyme in the small intestine. Since no inflamed areas or coccidial lesions were found in the small intestine, the cause of death is obscure.

Massive infections were present in the lambs dying from the effects of acute strongyloidiasis 13 to 18 days after infection (table 1). Lamb 513, which died 41 days after infection, had fewer parasites at death than lambs dying of uncomplicated strongyloidiasis. All the worms were recovered from the small intestines of 5 lambs at necropsy but small numbers were recovered from the abomasal contents of 3 lambs, in addition to large numbers from the small intestines.

The concentration of worms varied in different parts of the small intestine. This was determined by counting the number of worms found in different sections of the small intestine of lamb 527. The total worm count from this animal was 277,900. Of these worms, 89.5 per cent were located in the first 8 feet. The numbers recovered from the sections 0 to 2, 2 to 4, and 4 to 8 feet from the pylorus were 51,400, 92,950, and 104,350, respectively. The remaining worms were found in decreasing numbers in the next 17 feet of the small intestine. The numbers of worms recovered from the



Graph 3—Changes in the weights of lambs exposed to single applications of 100,000 or fewer larvae, as compared with the change in average weight of the uninfected controls (larvae in thousands).

sections 8 to 10, 10 to 15, and 15 to 25 feet from the pylorus were 17,700, 9,650, and 1,850, respectively. No worms were found in the remainder of the small intestine or in the large gut.

The proximal portion of the small intestine, therefore, appears to be the normal habitat of this parasite, at least in the early period of infection. The gross and histopathological changes in these infections confirmed the parasite's affinity for the anterior portions of the small intestine, as the damage was most severe in the first few feet of the small intestine.

The necropsy findings in the kid dying from strongyloidiasis were similar to those in the lambs. At necropsy of kid 275, much fluid was found in the abdominal and pleural cavities. The ventral borders of the lungs showed a condition suggestive of pneumonitis. The small intestine showed a typical catarrhal enteritis. It was inflamed in the upper portions, and its contents were liquid. About 25 per cent of the total larval dosage (75,950 worms) was recovered from the small intestine (table 1).

Kid 284 had partially recovered from the acute phase of its infection but, when it was killed 83 days after infection, it was emaciated. The blood was watery, but ascites was absent; the viscera and the intestinal contents otherwise appeared normal. Worms recovered from the small intestine totaled 6,970, or approximately 7 per cent of the original larval dosage.

DISCUSSION

Biology.—No difficulties were encountered in producing experimental infections of *S. papillosus* in the parasite-free lambs and kids by cutaneous application of larvae. The prepatent period of the parasite in these animals was nine days, which is in agreement with the work of Vegors [22] with calves and that of Garkavi [9] with lambs, on the same parasite.

Nonfatal infections of *S. papillosus* in lambs and kids usually ran their course in seven to eight weeks, although minor residual infections were retained for a longer period. Infected donor lambs maintained patent infections at variable, moderate to low, levels for as long as nine months, when moderately effective precautions were taken to prevent reinfection. Vegors [22] did not determine the maximum period of patency of *S. papillosus* in experimentally infected calves, but he did observe a five-month patency in 1 calf. The determination of maximum patency of Strongyloides is difficult, because extraneous infections may be acquired easily. However, in the case of *S. papillosus* in domestic ruminants, a small percentage of the parasites apparently can live for several months in these hosts, although the majority probably have a much shorter parasitic life.

The maximum or peak egg production of populations of *S. papillosus* occurred in lambs and kids about 15 to 18 days after infection, or early in the patent period. Vegors [22] reported that peak egg production occurred from 13 to 25 days after infection in calves. Therefore, *S. papillosus* apparently reaches maximum egg productivity at about the same time after infection in all three species of domestic ruminants. During patency, there was much fluctuation from day to day and week to week in the elimination of eggs in the feces.

No parasitic males of this parasite were found in any of the lambs and kids. This confirms the observations of most workers on Strongyloides spp. except those of Kreis [12] and Faust,[6] who reported parasitic males of *S. stercoralis* in dogs.

Pathogenicity.—It was significant that all the lambs and kids infected by large single exposures of *S. papillosus* larvae showed evidence of clinical and pathological effects. This definitely disproved the belief of Orlov [14] that this parasite is not a pathogen of sheep. The common clinical signs observed in the infected animals were anorexia, loss or retardation of weight, slight to moderate anemia, lassitude, difficult respiration, abnormal stools, and polyuria. The polyuria that developed in animals heavily infected with *S. papillosus* was accompanied by increased water consumption and by a strong odor of ammonia in the excessively wet cages. Polyuria also was reported to occur in human beings [4] infected with *S. stercoralis* and in calves [22, 24] infected with *S. papillosus.* The animals that developed polyuria were anemic, and ascites, hydrothorax, and other manifestations of edema were present at necropsy. It is probable that the lambs drank more water to compensate for the edema, which increased the output of urine. The observations on polyuria in Strongyloides infections were wholly qualitative, and further quantitative studies on this clinical condition are indicated.

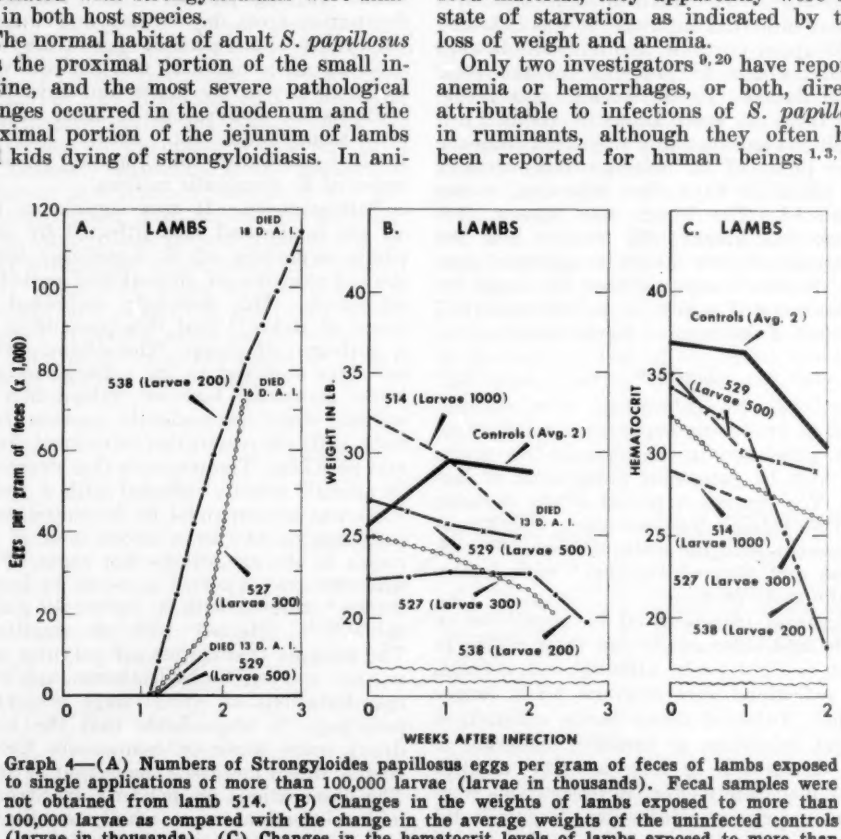
XUM

Of the 7 animals given single doses of 100,000 or more larvae, 6 died. Woodhouse [24] also reported a fatal case of strongyloidiasis in sheep and, later, Garkavi [9] reported several fatalities in lambs. Vegors[22] reported the deaths of young calves after infection was established with a single dose of 200,000 larvae of *S. papillosus,* whereas 4-month-old calves died after exposure to 1,000,000 or more larvae. Deaths of goats attributable to strongyloidiasis have not been reported in the literature. Oldham and Morgan [13] observed a herd of 80 goats for seven years and did not recover *S. papillosus* from any of the animals dying during that time. Nevertheless, the writer has demonstrated that lambs and kids apparently were equally susceptible to this parasite under certain experimental conditions. The clinical signs and pathological changes associated with strongyloidiasis were similar in both host species.

The normal habitat of adult *S. papillosus* was the proximal portion of the small intestine, and the most severe pathological changes occurred in the duodenum and the proximal portion of the jejunum of lambs and kids dying of strongyloidiasis. In animals with fatal cases, the intestinal mucosa was severely eroded and, in some, only the muscularis mucosa remained. Edema and leukocytic infiltration also were present. Larvae and adults were found to penetrate only to the muscularis mucosa, which apparently acted as a barrier. This agreed with the finding of Enigk [5] that larvae of *S. ransomi* did not burrow into the submucosa of the pig's intestine.

The profound pathological changes in the epithelium of the upper portion of the intestine adversely affected intestinal function. The impairment of digestion, absorption, and assimilation of nutrient materials was indicated by the abnormal fluid catarrhal exudate which usually filled the intestinal tract of severely affected animals instead of normal chyme. Although the rumens of some animals contained some food material, they apparently were in a state of starvation as indicated by their loss of weight and anemia.

Only two investigators [9, 20] have reported anemia or hemorrhages, or both, directly attributable to infections of *S. papillosus* in ruminants, although they often have been reported for human beings [1, 3, 10, 16]



Graph 4—(A) Numbers of Strongyloides papillosus eggs per gram of feces of lambs exposed to single applications of more than 100,000 larvae (larvae in thousands). Fecal samples were not obtained from lamb 514. (B) Changes in the weights of lambs exposed to more than 100,000 larvae as compared with the change in the average weights of the uninfected controls (larvae in thousands). (C) Changes in the hematocrit levels of lambs exposed to more than 100,000 larvae as compared with those occurring in the uninfected controls (larvae in thousands).

and dogs[2,8] infected with *S. stercoralis*, and for pigs[7,11,17,18] infected with *S. ransomi*. A possible cause of this anemia was the interference by the parasites with absorption of the minerals, vitamins, and other nutrients essential for normal hematopoiesis. This was suggested by Frickers,[7] who believed that enteritis in pigs caused by *S. ransomi* reduced the food intake and impaired absorption to the extent that hemoglobin synthesis was inhibited.

Damage to the lungs of infected animals apparently was limited to the early stages of infection, as lambs or kids necropsied 13 or more days after infection, with 1 exception, did not show abnormalities in these organs. The pathological changes in the lungs resulting from the effects of migrating larvae of *S. papillosus* will be discussed in a subsequent paper.

The author obtained no direct evidence that *S. papillosus*, during any phase of its parasitic cycle in lambs and kids, aided in the establishment of bacterial infections, as has been suggested.[5] Daily body temperatures of the lambs were normal throughout the period of observation, so it appears that pyrogenic viral or bacterial infections did not develop in these animals.

Summary

A total of 12 lambs and 3 kids were used in these studies. Eight lambs were exposed to single, cutaneous applications of 25,000 to 1,000,000 infective larvae of *S. papillosus*. Two kids were exposed to 100,000 and 300,000 larvae, respectively. Four lambs and 1 kid served as uninfected controls.

The observed signs of experimental, clinical strongyloidiasis were anorexia, loss or retardation in weight, slight to moderate anemia, lassitude, difficult respiration, abnormal stools, and polyuria. The 5 lambs exposed to 100,000 or more larvae died from 13 to 41 days after infection; the rapidity with which death occurred usually was directly related to the number of larvae to which the lambs were exposed. The kids apparently were as susceptible as lambs to experimental infections of this parasite; 1 of 2 infected kids died from the disease.

The prepatent period of the parasite in these animals was about nine days. The peak of egg production occurred 15 to 18 days after infection. Nonfatal infections usually ran their course in seven to eight weeks. Only parasitic females were found in the animals at necropsy.

Tissue damage in the animals dying from strongyloidiasis usually was confined to the duodenum and jejunum. A fluid, catarrhal exudate usually was found in the intestinal tracts, accompanied by severe enteritis. A tissue reaction involving leukocytic infiltration and edema also was present. Terminal lung lesions (pneumonitis and emphysema) were found in only 1 of these lambs with an essentially patent infection.

References

[1] Brau, P.: De l'*Anguillula intestinalis* en cochinchine et de son diagnostic hématologique. Bull. Soc. path. exot., *6*, (1913): 262–264.

[2] Burch, G. R., and Ehrenford, F. A.: Canine Strongyloidiasis. Vet. Med., *48*, (1954): 417–420.

[3] Coutinho, J. O., Croce, J., Campos, R., Amato Neto, V., and Fonseca, L. C.: Contribuição para o conhecimento da estrongiloidíase humana em São Paulo. Folia clin. et biol. (São Paulo), *20*, (1953): 141–176; *21*, (1954): 20–48, 93–120.

[4] Craig, C. F., and Faust, E. C.: Clinical Parasitology. 5th ed. Lea and Febiger, Philadelphia, Pa. (1951): 1032 pp.

[5] Enigk, K.: Pathogenität und Therapie des Strongyloidenbefalles der Haustiere. Monatsh. f. prakt. Tierheilk., *4*, (1952): 97–112.

[6] Faust, E. C.: Experimental Studies on Human and Primate Species of Strongyloides. II. The Development of Strongyloides in the Experimental Host. Am. J. Hyg., *18*, (1933): 114–132.

[7] Frickers, J.: Strongyloidosis bij het varken. Tijdschr. Diergeneesk., *78*, (1953): 279–303.

[8] Fülleborn, F.: Untersuchungen über den Infektionsweg bei Strongyloiden und Ankylostomum und die Biologie dieser Parasiten. Arch. Schiffs-u. Tropen-Hyg., *18*, (1914): 26–80.

[9] Garkavi, B. L.: Klinicheskaia kartina eksperimental'nogo strongiloidoza iagniat [Clinical Picture of Experimental Strongyloidiasis in Lambs]. Veterinariia, *33*, (1956): 37–38. Russian text.

[10] Hemsen: *Anguillula intestinalis* als Ursache einer schweren sekundären bezw. perniziösen Anämie. Deutsche med. Wchnschr., *49*, (1923): 83–85.

[11] Ippen, R.: Zur Pathogenität des *Strongyloides ransomi* unter besonderer Berücksichtigung seines Sitzes in der Schleimhaut der Darmwand. Arch. exptl. Vet.-Med., *7*, (1953): 36–57.

[12] Kreis, H. A.: Studies on the Genus Strongyloides (Nematodes). Am. J. Hyg., *16*, (1932): 450–491.

[13] Oldham, J. N., and Morgan, D. O.: Helminth Parasites Observed in a Herd of Goats Maintained at St. Albans, England. J. Helminthol., *12*, (1934): 39–46.

[14] Orlov, I. V.: K voprosu epizootologii strongiloidoza ovets i patogennom znachenii ego vozbuditelia. [The Epizootiology of Strongyloidiasis of Sheep and the Pathogenic Importance of Its Causal Agent]. Sovetsk. Vet., *15*, (1938): 74–79. Russian text.

[15] Roberts, F. H. S.: Ann. Rpt. Dept. Agric. & Stock, Queensland (1936–1937): 141–142.

[16] Sandground, J. H.: Some Studies on Susceptibility, Resistance, and Acquired Immunity to Infection with *Strongyloides Stercoralis* (Nematoda) in Dogs and Cats. Am. J. Hyg., 8, (1928): 507–538.

[17] Spindler, L. A., and Hill, C. H.: Death of Pigs

XUM

Associated with the Presence in the Heart Tissue of Larvae of *Strongyloides Ransomi*. Proc. Helminthol. Soc. Washington, *9*, (1942): 62–63.

[18] Spindler, L. A., Hill, C. H., and Zimmerman, H. E., Jr.: The Pathogenicity of *Strongyloides Ransomi*, the Intestinal Threadworm of Pigs. North Am. Vet., *24*, (1943): 475–486.

[19] Turner, A.: A Micro Method for Determination of Hemoglobin. Bull. U.S. Army M. Dept., *5*, (1946): 605–607.

[20] Turner, J. H.: Preliminary Report of Experimental Strongyloidiasis in Lambs. Proc. Helminthol. Soc. Washington, *22*, (1955): 132–133.

[21] Turner, J. H., and Wilson, G. I.: An Epizootic of Strongyloidiasis in Lambs. Vet. Med., *53*, (May, 1958): 242–243.

[22] Vegors, H. H.: Experimental Infection of Calves with *Strongyloides Papillosus* (Nematoda). Am. J. Vet. Res., *15*, (July, 1954): 429–433.

[23] Vegors, H. H., and Porter, D. A.: Experimental Cross Transmission of *Strongyloides Papillosus* in Ruminants. Proc. Helminthol. Soc. Washington, *20*, (1953): 91–92.

[24] Woodhouse, C. A.: Observations on Pathogenicity of Strongyloides Parasites in Ruminants. J.A.V.M.A., *113*, (Oct., 1948): 354–356.

Influence of Temperature on the Growth of Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution

F. ZUSCHEK, Ph.D.; R. P. HANSON, Ph.D.; C. A. BRANDLY, D.V.M.

Madison, Wisconsin

MOST RECENT STUDIES with Newcastle disease virus (NDV) in tissue cultures or in embryonating eggs employ an incubation temperature of 37.0 C. The chicken, which is a normal host for NDV, has a body temperature of 40.0 to 41.6 C. Since there is no reason to believe, a priori, that 37.0 C. is optimum for the growth of NDV in tissue cultures, this work was designed to study the kinetics of the development of infective and hemagglutinating particles of NDV at different temperatures.

MATERIALS AND METHODS

Virus.—With a few exceptions, when GB and B1 strains of NVD were used, the Roakin-NJ-1946 strain was used for the investigation of the effect of temperature on growth. The virus cultures were obtained from the repository for strains of NDV at the University of Wisconsin.

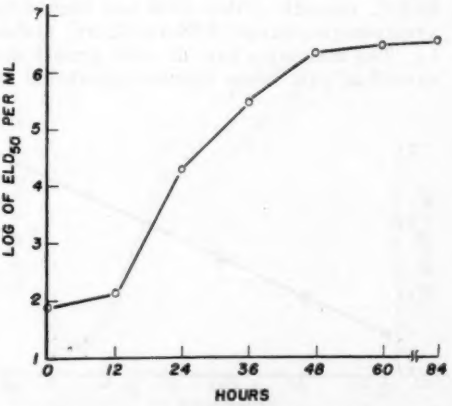
Eggs.—Fertile eggs from healthy White Leghorn chickens, free of pullorum disease, Newcastle disease, and infectious bronchitis, were used as tissues for growing and titrating the virus.

Virus Titrations.—Virus titers were based upon the lethality of the virus for 10- to 11-day embryonating chicken eggs. One tenth milliliter of a tenfold virus dilution, made in nutrient broth, was inoculated into the allantoic chamber of each of five eggs. The infected eggs were incubated at 37 C. and candled twice daily for seven days. Fluids from eggs showing questionable embryo deaths were tested for bacterial contamination and for the presence of hemagglutinins. Hemagglutinin titrations followed the method of Salk.[14] The 50 per cent embryo lethal dose (e.l.d.$_{50}$) was calculated by the method of Reed and Muench,[13]

Virus Cultures at Different Temperatures.—The method of culturing NDV in isolated, respiring chorioallantoic membranes (CAM) suspended in Tyrode's solution has been described by Scott *et al.*[18] and Zuschek *et al.*[19] Essentially, the method involves the aseptic removal of a CAM from a 10- or 11-day embryonating egg, then washing and suspending the membrane in 19 ml. of Tyrode's solution. Each membrane culture (tissue culture) was infected with 1 ml. of NDV containing $10^{2.3}$ e.l.d.$_{50}$. A minimal amount of virus was used initially to infect the CAM, so that the amount of residual virus in the solutions would be negligible. Culture fluid samples were removed at specified intervals and stored at −20 C. until virus titrations could be made. Only the amount of extracellular infective virus produced by the CAM was determined.

The different temperatures of incubation used in this work were 45, 42, 37, 33, and 25 C. The saline solution used for washing the CAM, as well as the Tyrode's solution, was incubated at each of the indicated temperatures for at least five hours prior to the addition of the CAM and the virus. Triplicate CAM cultures infected with NDV were incubated at each temperature.

The influence of temperature on hemagglutinin production in embryonating eggs was studied ac-



Graph 1—Growth of Newcastle disease virus (Roakin-NJ-1946) in chorioallantoic membranes suspended in Tyrode's solution and incubated at 37 C.

From the Department of Veterinary Science, University of Wisconsin, Madison. Dr. Zuschek is now with the Merck Sharp and Dohme Research Laboratories, West Point, Pa., and Dr. Brandly is dean, College of Veterinary Medicine, University of Illinois, Urbana.

Published with the approval of the director of the Wisconsin Agricultural Experiment Station as paper NS 248.

Based in part on a thesis submitted by the senior author to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree.

These studies were supported, in part, by funds from the Surgeon General's Office and by a grant from the research committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation.

XUM

cording to the following scheme. Twenty embryonating eggs, incubated at 37 C. for nine days, were placed in each of the following temperatures: 42, 37, 35, and 33 C., for 24 hours prior to inoculation with NDV. Virus was inoculated into ten eggs at each temperature, while the remaining eggs were inoculated with broth and served as controls. The virus used represented the product of culture in CAM at an incubation temperature corresponding to the incubation temperatures of the eggs. Both infected and control eggs were returned to their selected temperatures. The infected embryonating eggs that succumbed were chilled for 24 hours at 5 C. and 3 to 5 ml. of the extraembryonic fluid was harvested. The fluids were tested for bacterial contaminants and, if free, composites were titrated for virus infectivity and for hemagglutinin activity.

RESULTS

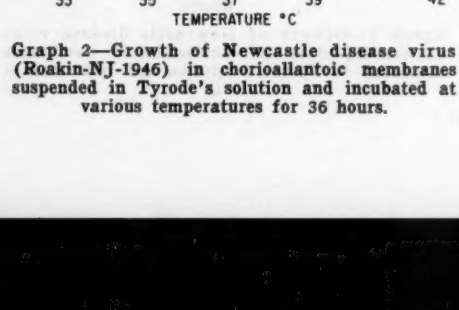
A typical growth curve of NDV (Roakin) in CAM, suspended in Tyrode's solution at 37 C., is shown (graph 1). The virus was in a log phase of growth after 12 hours. The growth of the virus reached a maximum stationary phase in 36 to 48 hours. These curves probably represent accumulative cycles of infection and liberation of infective virus at any given time, or leakage of virus and, consequently, accumulation. However, these accumulative cycles show a striking resemblance to typical growth curves of bacteria and will be described in those terms.

Growth of the Roakin strain was not obtained in CAM suspended in Tyrode's solution and incubated at a temperature of 25.0 C. or below; nor was there growth at 45.0 C. Growth of this virus was limited to a temperature range of 33.0 to 42.0 C. (table 1). The maximum rate of viral growth occurred in CAM tissue cultures incubated at 42.0 C. and decreased with a corresponding decrease in temperature. The growth of the virus apparently approached a maximum level at each of the temperatures in about 48 hours. These results are similar to those obtained in embryonating eggs by Bang,[1] who found that NDV grew well at temperatures of 35.0 to 41.0 C., with the maximum rate occurring at about 40.0 C. The fact that the maximum growth rate of the virus occurred in tissue cultures at 42.0 C. is not surprising, since this virus can reproduce in chickens whose body temperatures are about 41.6 C.

TABLE 1—Growth of Newcastle Disease Virus (Roakin-NJ-1946) in Chorioallantoic Membranes Suspended in Tyrode's Solution and Incubated at Various Temperatures

Temperature (C.)	Log of e.l.d.$_{50}$/ml. after indicated hours				
	0	12	24	36	48
45	1.2	NG*	NG	NG	NG
42	1.2	1.8	4.5	6.5	6.8
37	1.2	1.5	4.3	5.5	6.8
35	1.2	1.2	2.5	5.0	6.5
33	1.2	0.2	2.4	4.4	6.4
25	1.2	NG	NG	NG	NG

* NG = no virus growth obtained.



Graph 2—Growth of Newcastle disease virus (Roakin-NJ-1946) in chorioallantoic membranes suspended in Tyrode's solution and incubated at various temperatures for 36 hours.

A log-linear response is found if the amount of accumulative infective virus after 36 hours in CAM suspended in Tyrode's solution is plotted against the temperatures of incubation (graph 2).

Hemagglutinin activity was not obtained from 48 hours' growth of the virus in any of the tissue cultures incubated at all the temperatures. This was strange, since infective virus yields from the CAM closely approached viral levels from growth in embryonating eggs. The possibility of a hemagglutinin inhibitor was considered. However, the use of dialysis or various solvents, or both, failed to increase the hemagglutinin titers of negative samples above a 1:5 dilution. These findings differ with those of Scott *et al.*,[18] who obtained positive hemagglutinin activity in titers of over 2 logs with the Roakin strain of NDV in CAM suspended in Tyrode's solution and incubated at 37 C. for 48 hours. Lederberg [9] reports that Burnet considers the soluble buffers of Tyrode's solution to be incapable of providing an adequate buffer system for hemagglutinin production.

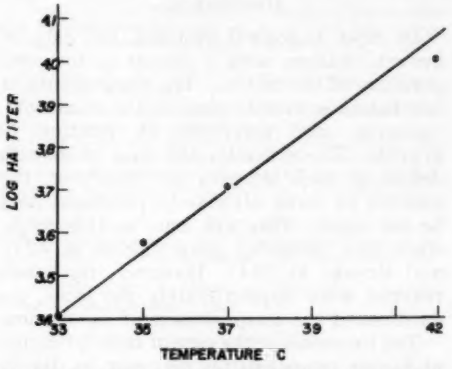
Although hemagglutinin activity of the Roakin strain was not obtained from growth in tissue cultures, this was not true when the virus was cultured in embryonating

eggs incubated at different temperatures. The maximum hemagglutinin titer was obtained from infected eggs which had been incubated at 42 C.; the minimum titer was obtained from infected eggs at 33 C.

A log-linear response is obtained when the logs of the hemagglutinin titers are plotted against the temperatures of incubation of the infected eggs (graph 3). However, the e.l.d.$_{50}$ of fluids obtained from these eggs after death of the embryos showed no significant differences. This is not in disagreement with the results obtained from the growth of the virus in tissue cultures, since the virus grew in the eggs for three or more days. This was probably time enough for the NDV to reach its maximum titer, irrespective of the temperature of incubation. Had the growth rates of the virus in eggs at different temperatures been followed, a pattern similar to that obtained in the tissue cultures might have been found. It had been noticed, however, that embryonating eggs infected with virus and incubated at 42 C. died 12 to 18 hours sooner than infected eggs incubated at 33 C. There was a progressive decrease in the embryo death times as the temperatures of incubation rose from 33 to 42 C. None of the embryos in the control eggs, at any of the temperatures used, died during the periods of observation.

Further studies were made to determine if differences in the growth patterns of three strains of NDV (GB, highly pathogenic for chickens of any age; Roakin, moderately pathogenic for young chickens; and B1, almost apathogenic for chicks) could be obtained in tissue culture. Experiments were also designed to determine whether these three strains would grow at different rates at different incubation temperatures.

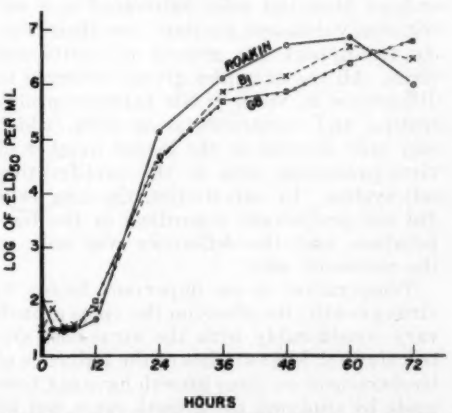
No significant differences in the growth patterns of GB, Roakin, and B1 strains of NDV were obtained in CAM tissue cultures incubated at 37 C. (graph 4). All three strains had a latent period of about six to eight hours and were in their log phase of growth up to about 36 to 48 hours. The growth rates did not differ from one another when the tissue cultures in which the viruses were growing were incubated at different temperatures (graph 5). A log-linear response of growth of these three strains at different temperatures (graph 5)



Graph 3—Hemagglutinin production in embryonating eggs infected with Newcastle disease virus (Roakin-NJ-1946) and incubated at various temperatures.

is almost identical to that obtained with the Roakin strain alone (graph 2).

The three strains of NDV (GB, Roakin, and B1) differ from one another in many respects, especially in their lethality for chicks, as reported by Hanson.[5] However, their growth rates and patterns in CAM were similar. Differences between the three strains may be due to variability in their tissue tropisms or to differences in their responses to the host's defense. Differences were not obtained between these strains, possibly because only one type of tissue was used and only infectious virus production was being measured.



Graph 4—Growth patterns of three strains of Newcastle disease virus (Roakin, B1, and GB) in chorioallantoic membranes suspended in Tyrode's solution and incubated at 37 C.

XUM

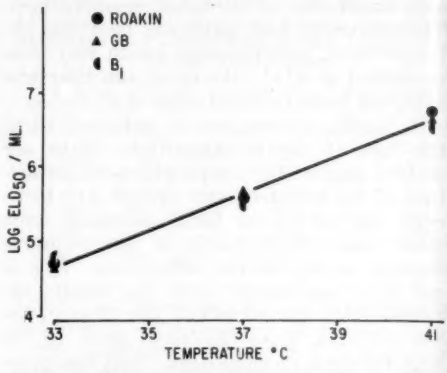
DISCUSSION

In most biological systems, the rate of growth changes with a change in the temperature of incubation. The temperature of incubation is usually classified as minimum, optimum, and maximum in relation to growth. Theoretically, the rate of growth differs at each temperature; however, the amount of virus ultimately produced may be the same. This was true in this work, since NDV (Roakin) grew fastest at 42 C. and slowest at 33 C. However, the titers reached were approximately the same, regardless of the temperatures of incubation.

The increases in the rate of multiplication at higher temperatures may not be due to something inherent in the virus, but might be caused by an increase in metabolic activity of the host cells (cells of CAM).

Hershey and Bronfenbrenner,[8] and Northrop [10] have shown that the metabolism and the growth rates of bacteria have a dramatic influence on the amount of bacteriophage produced per cell. This modification in yield also has been demonstrated with animal viruses. Fox [2] found that higher yields of yellow fever virus were obtained from actively proliferating tissue, such as muscle, than from slow growing tissue, such as brain. Scherer [17] found that herpes simplex virus grew better in proliferating tissue than in maintenance tissue cultures. Plotz and Reagan [12] asserted that proliferating cells of the chicken embryo were essential for the growth of rabies virus. Hare and Morgan [7] have shown that chicken embryo fibroblast cells, cultivated in a nutritionally deficient medium, lose their ability to support the growth of psittacosis virus. All the examples given [7] referred to differences in virus yields between proliferating and nonproliferating cells, which may only depend on the larger number of virus-producing cells in the proliferating cell system. In our studies, the CAM cells did not proliferate, regardless of the temperature, and the difference was only in the metabolic rate.

Temperature is an important factor in virus growth; its effects on the virus growth vary considerably with the virus-host system studied. Most studies of the influence of temperatures on virus growth have not been made by studying the growth rates, but by determining the quantity of virus produced after a period of incubation at a given temperature. Greiff and Pinkerton [4] found



Graph 5—Growth rates of three strains of Newcastle disease virus (Roakin, B1, and GB) in chorioallantoic membranes suspended in Tyrode's solution and incubated at different temperatures for 36 hours.

that the highest titer of influenza virus was obtained at 37 C., while the lowest titer was obtained at 40 C. Pearson [11] found that the virus of mouse encephalomyelitis, grown in tissue cultures, produced equivalent titers at 31 to 37 C., but that the titers did not exceed 10^2 at 25 and 38 C., indicating a narrow range of temperatures in which this virus could grow.

Most viruses are grown in tissue cultures at 37 C., although their optimum temperature for growth may be slightly above or below this. Sanders [15] and Sanders and Alexander [16] found that higher titers of the viruses of lymphogranuloma venereum, equine encephalomyelitis, and epidemic keratoconjunctivitis could be obtained at room temperature, rather than at 37 C.

Temperature not only modifies virus growth rates and titers, but can also influence the lethality of the virus to its host. Newcastle disease virus (Roakin) would kill embryonating eggs much faster at 42 0 C. than at 37.0 or 33.0 C. Greiff [3] states that the lethal effect of influenza virus on embryos was least marked at 40.0 C. and that the lowest and most variable infectivity titers were found at this temperature. The highest titer and greatest lethal effects were obtained at 37.5 C. This is inconsistent with our study, but may be due to the greater lability of influenza virus, as compared with the stability of NDV. Hanson and Sinha [6] have found that the lethal death time (time required for a given concentration of virus

to produce death) of chicken embryos infected with NDV has a linear function with temperature.

SUMMARY

The growth rates and growth patterns of Newcastle disease virus (NDV) were studied in chorioallantoic membranes suspended in Tyrode's solution and incubated at various temperatures. The virus grew best in tissue cultures incubated at 42 C.

Growth of NDV (Roakin-NJ-1946) was limited to incubation temperatures between 33 and 42 C. The production of hemagglutinins in embryonating eggs infected with this virus followed a log-linear response to temperature.

The growth patterns of strains GB, B1, and Roakin of NDV, in tissue cultures incubated at 37 C., did not differ significantly from one another. Pronounced differences in the growth rates of these three virus strains at different temperatures of incubation were not demonstrable. The production of virus in tissue culture followed a log-linear response to temperature after 36 hours of incubation.

Hemagglutinin production was not demonstrable in tissue cultures of any of the three virus strains.

References

[1] Bang, F. B.: Studies on Newcastle Disease Virus. I. An Evaluation of the Method of Titration. II. Behavior of the Virus in the Embryo. J. Exptl. Med., *88*, (1948): 233–249.

[2] Fox, J.: The Cultivation of Yellow Fever Virus. I. Factors Influencing the Multiplication of 17 D Virus in Tissue Culture. Am. J. Hyg., *46*, (1947): 1–20.

[3] Greiff, D.: The Chick Embryo in Biological Research, the Metabolic Interactions of Intracellular Parasites and Embryonated Eggs. Ann. New York Acad. Sci., *55*, (1952): 254–266.

[4] Greiff, D., and Pinkerton, H.: Effect of Temperature and Para-Aminobenzoic Acid on the Growth and Oxygen Uptake of Embryonated Eggs. Fed. Proc., *10*, (1951): 356.

[5] Hanson, R. P.; University of Wisconsin, Madison: Unpublished data, 1954.

[6] Hanson, R. P., and Sinha, S. K.; University of Wisconsin, Madison: Unpublished data, 1953.

[7] Hare, J. D., and Morgan, H. R.: Studies on the Factors Essential to the Initiation and Maintenance of Multiplication of Psittacosis Virus (6 BC Strain) in Deficient Cells in Tissue Culture. J. Exptl. Med., *99*, (1954): 461–479.

[8] Hershey, A. D., and Bronfenbrenner, J.: Bacterial Viruses: Bacteriophages. In "Viral and Rickettsial Infections of Man." J. B. Lippincott Co., Philadelphia, Pa., 1948.

[9] Lederberg, J.; University of Wisconsin, Madison: Personal communication, 1958.

[10] Northrop, J. H.: Growth and Phage Production of Lysogenic *B. Megatherium*. J. Gen. Physiol., *34*, (1951): 715–735.

[11] Pearson, H. E.: Factors Affecting the Propagation of Theiler's GD V11 Mouse Encephalomyelitis Virus in Tissue Cultures. J. Immunol., *64*, (1950): 447–453.

[12] Plotz, H., and Reagan, R.: *In Vitro* Cultivation of the Street Virus of Rabies. Science, *95*, (1942): 102–104.

[13] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[14] Salk, J. E.: A Simplified Procedure for Titrating Hemagglutinating Capacity for Influenza Virus and the Corresponding Antibody. J. Immunol., *49*, (1944): 87–98.

[15] Sanders, M.: Studies on the Cultivation of the Virus of Lymphogranuloma Venereum. J. Exptl. Med., *71*, (1940): 113–128.

[16] Sanders, M., and Alexander, R. C.: Epidemic Keratoconjunctivitis. I. Isolation and Identification of a Filterable Virus. J. Exptl. Med., *77*, (1943): 71–96.

[17] Scherer, W. F.: The Utilization of a Pure Strain of Mammalian Cells (Earle) for the Cultivation of Viruses *in Vitro*. I. Multiplication of Pseudorabies and Herpes Simplex Viruses. Am. J. Path., *29*, (1953): 113–137.

[18] Scott, G. R., Hanson, R. P., and Brandly, C. A.: A Simplified Procedure for the *in Vitro* Propagation of Newcastle Disease Virus. Proc. Book, AVMA (1953): 312–315.

[19] Zuschek, F., Hanson, R. P., and Brandly, C. A.: Factors Influencing the Growth of Newcastle Disease Virus in Isolated Chorioallantoic Membranes. Am. J. Vet. Res., *19*, (Jan., 1958): 191–195.

XUM

Titration of Newcastle Disease Virus in Tissue Culture

D. DURAND, M.S., and A. EISENSTARK, Ph.D.

Manhattan, Kansas

RECENT ADVANCES in tissue culture techniques have given rise to new opportunities in the study of viruses. Since cell destruction as a result of virus multiplication may be observed in the test tube, it is possible to titrate the virus infectivity and the neutralizing ability of serum.

Salk *et al.*[7] developed a simplified method for polio virus titration, based on inhibition of the metabolic activity of cells; virus multiplication (or its inhibition by antiserum) was detected by the degree of color change of phenol red as a result of acid production by viable cells.

The purpose of this report is to describe a modification of the Salk titration method as applied to the titration of Newcastle disease virus and its specific antiserum.

MATERIALS AND METHODS

The California strain (11914) and the L.W. strain of Newcastle disease virus (NDV) were used in this study. Stock cultures of both strains were prepared by inoculation into the allantoic cavities of 10-day-old chicken embryos. After death of the embryos, the allantoic fluids containing virus were harvested and 2-ml. amounts were sealed in glass vials, quick frozen, and stored in a dry-ice box. The 50 per cent embryo infectivity ($e.i._{50}$) end points and hemagglutination (HA) titers were determined periodically on all stock fluids to assure high titer stocks.

Hanks' balanced salt solution[3] (BSS) was used for virus and serum dilutions. The maintenance medium used was medium 199 (described by Morgan *et al.*[5]), supplemented with low concentrations of serum and embryo extract.

Chicken embryo extract was prepared from 10-day-old embryos by grinding whole embryos in a Waring blendor with an equal volume of BSS, centrifuging the resulting suspension at $20{,}000 \times g$ for two hours, and sterilizing the supernatant fluid by filtration through a Selas 03 filter. The chicken embryo extract was stored at −20 C. Hemagglutination, hemagglutination-inhibition, and infectivity titrations were performed by standard procedures described by Cunningham.[2]

Tissue Culture Procedures.—Trypsinization of chicken embryo tissues were performed either at 37 C. for 15 minutes[6] or at 4 C. for approximately 18 hours.[1] The procedure for cold trypsinization differed from trypsinization at 37 C. only in time and temperature. Ten 10-day-old chicken embryos were decapitated and washed twice with 30 ml. of BSS, expressed through a 10-ml. syringe into 10 ml. of 0.5 per cent trypsin solution, and refrigerated at 4 C. for approximately 18 hours. At the end of trypsinization, an equal volume of BSS was added, and the suspension was filtered through two layers of cheesecloth and centrifuged at 500 r.p.m. in a horizontal centrifuge for 20 minutes. The cells were then washed once and counted in a hemocytometer.

HeLa cells, from a strain of human epithelium originally cultured from a cervical carcinoma,[8] were maintained in stock culture by growing them in a medium consisting of 80.0 per cent Difco 199, 10.0 per cent human serum, and 10.0 per cent horse serum. Trypsinization was carried out at 37 C. for 30 minutes, using a 0.5 per cent trypsin solution.

General Titration Procedures.—Replicate cultures (table 1) were prepared in 13- by 50-mm. flat-bottomed screw-capped vials. The use of flat-bottomed vials was preferred, as the cells were able to spread evenly over the flat surface.

Known concentrations of the cells were suspended in the medium for the test, and a magnetic stirrer was used to keep the cells suspended uniformly throughout the medium. A constant volume (usually 1 ml.) of the cell suspension was delivered to each of the vials by an automatic pipette. One tenth milliliter of the virus was added and the vials were stoppered with No. 00 white rubber stoppers. Incubation was carried out at 35 C. with the cultures in an upright position.

To have a standard for purposes of comparison, it was first necessary to determine the required cell concentration needed to show acid production, as seen by the change of phenol red to a yellow color within a reasonable length of time. It was found that 1×10^6 chicken embryo cells per vial containing 1.0 ml. of medium plus 0.1 ml. of normal allantoic fluid would cause a shift from red to yellow within 72 hours at 35 C., allowing the test to end. As a control, normal allantoic fluid was tested to verify that any change in cell metabolism was due to virus and not to the allantoic fluid in which the virus was suspended. One milliliter of medium, consisting of Difco 199, 2 per cent serum, and 1 per cent embryo extract and containing 1×10^6 chicken embryo cells, was added to each of 33 vials. Normal allantoic fluid was diluted tenfold, serially 10^{-1} to 10^{-8}, in BSS and 0.1 ml. from each dilution was inoculated into each of five vials per dilution. The three control vials received 0.1 ml. of BSS. The experiment ended after 72 hours and vials were

Contribution No. 335, from the Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan.

checked for a uniform color change from red to yellow.

In certain experiments, dilute methylene blue and varying concentration of 2,3,5-triphenyl-2H-tetrazolium chloride were substituted for phenol red to test for their possible value as indicators.

Titration of Immune Newcastle Disease Virus Serums in Tissue Culture.—The antigen concentration was determined by titrating NDV, utilizing the color end point method previously described. A suspension of virus, diluted to 1×10^5 particles per milliliter, was found to give consistent positive results, as seen by the retention of red color in all inoculated cultures. This suspension was used as the antigen.

The culture vials were prepared by adding 1.0 ml. of freshly trypsinized cells to each vial, at a concentration of 1×10^6 cells per milliliter. The immune NDV serum was then diluted twofold serially, 1:5 to 1:2,560 (table 2, column 1). One tenth milliliter of each serum dilution was added to five vials. One tenth milliliter of the NDV antigen was added to each of 40 vials containing the dilution of immune serum and cells and to ten vials containing only cells, the latter acting as the virus control (table 2, column 6). To ten vials containing only immune serum and cells (serum control, table 2) and to ten vials containing only antigen and cells (virus control, table 2), 0.1 ml. of BSS was added to bring the volume to 1.2 ml. The vials were then sealed and incubated at 33 C. for five days. The neutralization titer of the serum was determined by comparison of the color change in the serum and the virus control vials.

RESULTS

Results obtained from NDV titration series are presented (table 1). The end point of tissue culture titration (calculated from column 6) is found to be $10^{-7.83}$. When HeLa cells were substituted for chicken embryo fibroblasts as the susceptible cells, the results were comparable to those listed, but with slightly lower titers. This was probably due to the more rapid metabolism of the HeLa cell, causing a change of pH before the virus could destroy the cell.

In a search for an indicator that might be more satisfactory than phenol red, certain other reagents were tested. A titration series was set up without phenol red in the medium. When incubation ended, 0.1 ml. of 0.05 per cent methylene blue solution was added to each vial. A slight color difference from blue to light blue-green was observed, corresponding to virus dilution. This color difference was not as distinct as in the control series, which received phenol red. When methylene blue was added to those vials which contained phenol red, the end point (as indicated by the color change from blue-green to green, corresponding to the color change from red to yellow for phenol red) was clearer than with either indicator alone. The 2,3,5-triphenyl-2H-tetrazolium chloride was tested for use as an indicator but was found to be toxic to the cells.

TABLE 1—Titration of Newcastle Disease Virus by Color End Point Method in Replicate Tissue Cultures of Chicken Embryo Fibroblasts

Dilution	Series					
	1	2	3	4	5	6
10^{-1}	R	R	R	R	R	5/5*
10^{-2}	R	R	R	R	R	5/5
10^{-3}	R	R	R	R	R	5/5
10^{-4}	R	R	R	R	R	5/5
10^{-5}	R	R	R	S	R	5/5
10^{-6}	S	S	Y	Y	Y	2/5
10^{-7}	Y	Y	Y	Y	Y	0/5
10^{-8}	Y	Y	Y	Y	Y	0/5
10^{-9}	Y	Y	Y	Y	Y	0/5

* Numerator = No. of vials showing virus effect (red); denominator = No. of vials used.

Scoring was based on comparison with color changes in control tubes. R = red color, S = salmon color, Y = yellow color. The red color indicated no color change and, therefore, that enough virus was present to kill cells before acid was produced.

Since it was demonstrated that NDV could be titrated, using the color end point method, neutralization tests were performed to determine whether this method was sensitive enough to be used for the testing of immune serums.

The results, obtained from a titration series using specific immune serum from inoculated chickens, are listed (table 2). The 1:5 dilution of serum was observed to be toxic to the cells and was discarded. The results were similar for three separate experiments.

DISCUSSION

Although the replicate tissue culture titration technique is less sensitive than when NDV is titrated in eggs, there are at least three advantages to the use of tissue culture in titration over the use of the chicken embryo infectivity method: (1) The system can be freed of all antibodies by repeated washing of the cells used; (2) tissue culture is a more uniform system; and (3) the cost of tissue culture titration, once established on a routine basis, would be much less than embryo infectivity titration.

The titration procedure using color change in replicate tissue cultures was found to be sensitive enough for use in neutralization tests. The neutralization test developed used the constant virus-varying serum procedure and proved to be a dependable method for titration of the protective capacity of immune serum. Although the

XUM

TABLE 2—Neutralization Test of Newcastle Disease Virus Immune Serums in Tissue Culture

Serum dilution	Virus added (replicates) 1	2	3	4	Serum control	Virus control	Results
5	...	...	...	...		R	...
10	Y	Y	Y	Y	Y	R	4/4*
20	Y	Y	Y	Y	Y	R	4/4
40	Y	Y	Y	Y	Y	R	4/4
80	Y	Y	Y	Y	Y	R	4/4
160	S	R	S	Y	Y	R	1/4
320	R	R	R	R	Y	R	0/4
640	R	R	R	R	Y	R	0/4
1,280	R	R	R	R	Y	R	0/4
2,560	R	R	R	R	Y	R	0/4

* Numerator = No. of vials showing neutralization effect (yellow); denominator = No. of vials used.
R = red color, S = salmon color, Y = yellow color. The red color indicated that enough virus was present to kill cells before acid was produced.

hemagglutination-inhibition (HI) test is available for work with NDV, a titration procedure using infectivity could be from 10^5 to 10^6 times more sensitive than a method using the HI test.[4] The HI test is a measure of the serum's ability to inhibit agglutination of erythrocytes. The tissue culture neutralization test is a measure of the serum's ability to inhibit infection of susceptible cells and, therefore, is a more direct answer to the specific question that is raised about the property of the antiserum; *i.e.*, its ability to neutralize the infectious property of the virus.

Although a combination of methylene blue and phenol red gave a slightly better end point, the added manipulations do not seem to warrant the routine addition of methylene blue as an indicator.

The results indicate that it should be possible to extend this method of titration to include avian viruses, such as infectious bronchitis or laryngotracheitis, which do not agglutinate erythrocytes but are capable of upsetting cell metabolism in some tissue culture systems.

SUMMARY

A replicate tissue culture system for Newcastle disease virus (NDV) titration was developed. This titration method depends on color change of pH indicators, due to the different degrees of metabolism of infected and noninfected tissue cells. Using this method, it was possible to titrate NDV and anti-NDV serums with reproducible results.

References

[1] Bodian, David: Simplified Method of Dispersion of Monkey Kidney Cells with Trypsin. Virology, *2*, (1956): 575–576.

[2] Cunningham, C. H.: A Laboratory Guide in Virology. 2nd ed. Burgess Publishing Co., Minneapolis, Minn., 1953.

[3] Hanks, J. H., and Wallace, R. E.: Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration. Proc. Soc. Exptl. Biol. & Med., *71*, (1949): 196–200.

[4] Luria, S. E.: General Virology. John Wiley and Sons, New York, N.Y. (1953): 253.

[5] Morgan, J. F., Morton, H. J., and Parker, R. C.: Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on a Synthetic Medium. Proc. Soc. Exptl. Biol. & Med., *73*, (1950): 1–8.

[6] Rous, P., and Jones, F. S.: A Method for Obtaining Suspensions of Living Cells from the Fixed Tissues, and from Plating Out Individual Cells. Proc. Soc. Exptl. Biol. & Med., *13*, (1915): 73.

[7] Salk, J. E., Younger, J. S., and Ward, E. N.: Use of Color Change of Phenol Red as an Indicator in Titrating Poliomyelitis Virus or Its Antibody in a Tissue Culture System. Am. J. Hyg., *60*, (1954): 214–230.

[8] Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the Propagation *in Vitro* of Poliomyelitis Viruses. IV. Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix. J. Exptl. Med., *97*, (1953): 695–710.

Tissue Culture of Avian Visceral Lymphoid Tumors and *in Vitro* Serial Passage of the Virus

OLIVE S. DAVIS, Ph.D., and DONALD P. GUSTAFSON, D.V.M., Ph.D.

Lafayette, Indiana

NEOPLASTIC TISSUES from chickens have been studied *in vitro* for many years. Carrel and Burrows[2] cultured Rous sarcoma as early as 1910, and there are now many reports on the culture of Rous, Fujinami, and other fowl sarcomas *in vitro*. Cultures of the virus of leukosis also have been reported by many workers.[1, 3-5, 7, 8, 12-14, 16, 18, 19]

In 1937, Ludford[10] described the production of tumors in cultures of normal cells treated with filtrates of Rous or Fujinami sarcomas; in 1938, Ruffilli[15] described the infection of normal chicken embryo heart cultures with erythroleukemic avian blood.

Until recently, *in vitro* studies of avian lymphoid leukosis had not been reported. At the 1957 meeting of the Poultry Science Association, two reports were made on the cultivation of the virus of avian visceral lymphomatosis (AVL) in normal chicken embryo tissues.[6, 17] The work reported here apparently was being done at about the same time, but independently and with a different approach.

The purpose was to explore the possible usefulness of *in vitro* technique in other research with this disease. The immediate objectives were: (1) to observe the differences between cultures of normal and neoplastic tissues; (2) to detect possible changes in normal cultures subjected to virus-containing materials; and (3) to determine if serial passage of the virus would be possible in cultures of spleens from 19-day-old chicken embryos, as shown by the inoculation of susceptible chickens with supernatant fluid from the tissue cultures. The approach was to culture tissues from chickens affected with AVL and to use supernatant fluids from these cultures to initiate serial passages of the virus in cultures of normal chicken embryo spleens.

MATERIALS AND METHODS

Source of Tumors for Tissue Culture.—In all trials, the tumor materials used were obtained from chickens affected with AVL in serial passage experiments using strain RPL 12.* Normal tissues were obtained from control chickens of the same age and lots. In five trials, primary explants from the following tissues were cultured: trial 1, neoplastic pectoral muscle, liver, spleen, and kidney; trial 2, neoplastic pectoral muscle and normal pectoral muscle; trial 3, neoplastic pectoral muscle; trial 4, neoplastic pectoral muscle, liver, and spleen and normal pectoral muscle, liver, spleen, and kidney; trial 5, neoplastic pectoral muscle, and spleen.

Tissue Cultures.—Methods previously described by Gustafson and Pomerat[9] were used. Primary explants were cut approximately 1.0-mm. square. Five explants were mounted in a chicken plasma and chicken embryo-extract clot on each 12- by 50-mm. glass cover slip, and two cover slips were placed back to back in a 16- by 150-mm. Pyrex culture tube stoppered with cotton. After these were allowed to remain undisturbed at room temperature for about three hours, 2 ml. of a nutrient medium was added to each tube. The tubes were then restoppered with latex rubber stoppers and placed in a roller drum revolving at about 12 rotations per hour in an incubator at 35 C. The fluid medium used in trials 1 through 3 consisted of 50 per cent human ascitic fluid, 45 per cent Gey's solution containing 0.2 mg. per cent phenol red, 5 per cent chicken embryo extract, and 1,000 units of penicillin per milliliter. In trials 4 and 5 a second medium was used, consisting of eight parts Gey's balanced salt solution, one part chicken serum, and one part bovine lactalbumin hydrolysate with phenol red added, but no antibiotic.

Eight to 17 (usually 10 to 12) tubes were prepared from each tissue. With each tube containing ten explants, about 100 explants of each tissue cultured were available for observation. Every two or three days during the maintenance of the cultures, the fluid medium was withdrawn by pipette and replaced with fresh medium. At various intervals, cultures were observed by phase microscopy. Some were photographed while liv-

From the Department of Veterinary Science, Purdue University, Lafayette, Ind. Published as journal series paper No. 1282 of Purdue University Agricultural Experiment Station.

Presented before the Section on Poultry, Ninety-Fifth Annual Meeting, American Veterinary Medical Association, Philadelphia, Pa., Aug. 18–21, 1958.

These studies were supported in part by a grant from the Indiana Elks Cancer Research Fund, administered by the Purdue Research Foundation.

* Avian visceral lymphomatosis of Regional Poultry Laboratory strain 12.

XUM

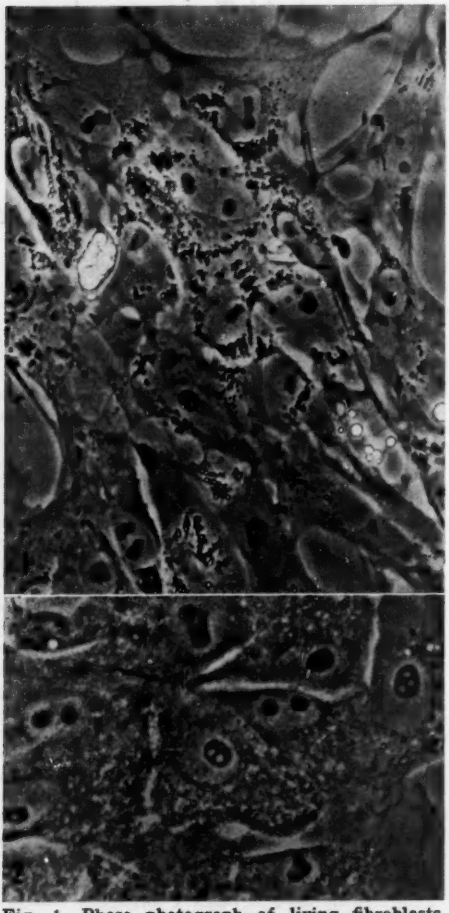


Fig. 1—Phase photograph of living fibroblasts. Normal 19-day chicken embryo spleen cultures at 13 days. x 420.

Fig. 2—Phase photograph of living fibroblasts. Third serial passage of avian visceral lymphomatosis virus on 19-day chicken embryo spleen cultures at 13 days. (notice granularity of cytoplasm as contrasted with homogeneity in figure 1). Vacuolization of the nucleoli is present in both figures.

ing; others were fixed in absolute methyl alcohol, stained by Jacobson's technique or with hematoxylin and phloxine stains, and mounted.

Primary explants of spleens from normal 19-day-old embryonating chicken eggs were prepared similarly for the serial passage of the virus.

Serial Passage of the Virus.—At the time of preparation of the spleen explants, 0.2 ml. of the virus-containing supernatant fluids harvested from cultures of tumors of chickens were added to 1.8 ml. of the fluid medium of some of the spleen explants. Others were supplied with 0.2 ml. of supernatant fluid from normal tissues and 1.8 ml. of medium. Supernatant fluids harvested from the virus-treated cultures were added in series to succeeding preparations of normal chicken embryo spleens at three-, five-, and seven-day intervals.

Control and virus-treated cultures were observed under bright field and phase conditions of microscopy. Photographs were taken under phase conditions (fig. 1, 2); some cultures were fixed, stained, and mounted prior to being photographed.

Chicken Inoculations.—In all trials, supernatant fluids harvested from cultures of chicken tumor tissues were inoculated into pectoral muscles of chickens, 20 to 38 days old, to test for the presence of the virus (table 1).

Supernatant fluids from some of the passage cultures of chicken embryo spleens also were inoculated intramuscularly into groups of chickens, 19 to 22 days old, to test for the continued presence of the virus (table 2). In two of these trials, since no cases of the disease had appeared after 38 days, one half of each inoculated group was reinoculated with ground tumor suspended in saline solution. The tumor was excised from a chicken affected with AVL, strain RPL 12.

RESULTS

Chicken Tissue Cultures.—In all cultures of tumorous tissues, with the exception of the liver explants in the first trial, migration of lymphoid cells was observed as

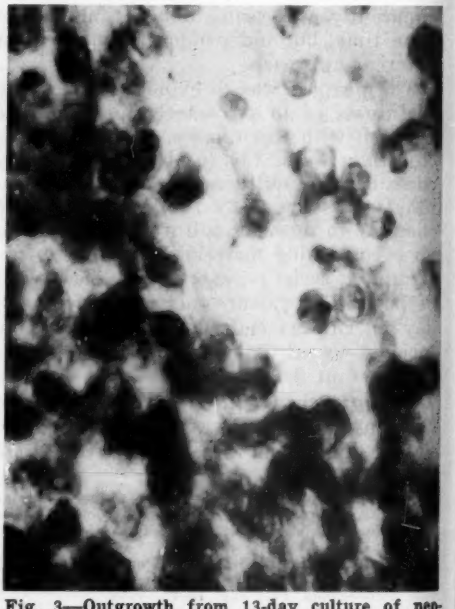


Fig. 3—Outgrowth from 13-day culture of neoplastic pectoral muscle from a chicken. Outgrowth is composed primarily of lymphoblasts. Jacobson's stain; x 1066.

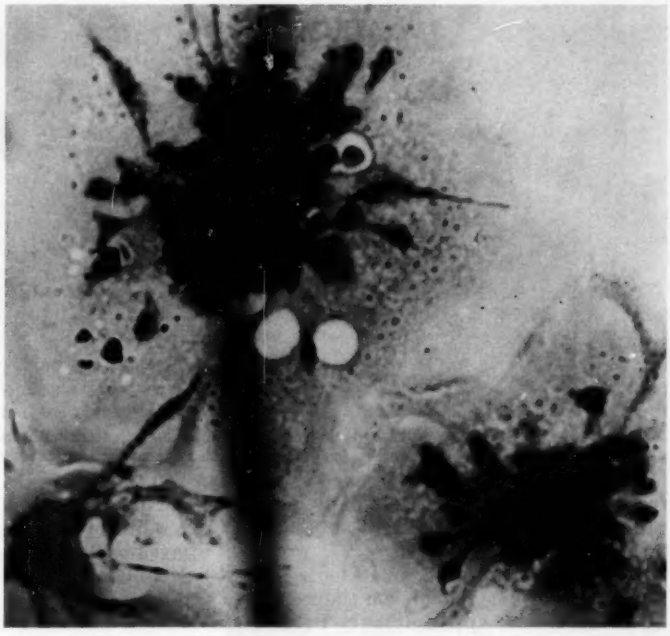


Fig. 4—Thirteen-day culture of spleen from chicken with avian visceral lymphomatosis. Giant cells appear to have germinal centers from which nuclei migrate peripherally. These formations characterize the cultures at this age following the initial massive migration of lymphoblasts. Jacobson's stain; x 850.

early as three or four hours after culture, fibroblastic outgrowth was apparent within about 24 hours, and massive proliferation of lymphoid tumor cells was apparent within 48 hours (fig. 3–5). In trial 4, when the second medium was used, outgrowth

Fig. 5—Five-day culture of spleen from a chicken affected with avian visceral lymphomatosis. Notice contrast between fibroblast outgrowth on the left and the transitional lymphoblastoid cells on the right in the early phases of giant cell development. Jacobson's stain; x 194.

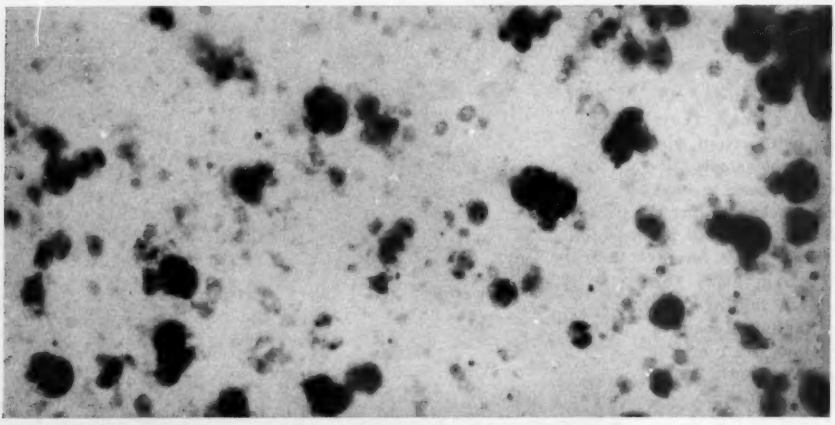


Fig. 6—Thirteen-day culture of liver from a chicken affected with avian visceral lymphomatosis. Notice the packets of lymphoblasts in the outgrowth. Hematoxylin and phloxine; x 224.

from the tumorous liver explants was as rapid and extensive as from the other neoplastic tissues (fig. 6).

Outgrowths appeared much more slowly in cultures of normal chicken tissues; after 48 hours it was apparent only in normal spleen cultures. After seven days, outgrowth was present also in normal liver and kidney explants, although still slight as compared with tumor explants of the same age. Outgrowth still was not apparent in normal muscle explants.

The results of tests for the presence of the virus of AVL in chickens by intramuscular inoculations of supernatant fluids harvested from primary explants of various tissues manifesting neoplasia (table 1) showed that a high incidence of lymphomatosis developed in most of the inoculated groups, usually with lymphoid tumors appearing in the pectoral muscle at the site of inoculation. Even a few cases developed in the group inoculated with a Selas 03 filtrate of this supernatant fluid and in the group inoculated with supernatant fluid held frozen for three months. Some of the chickens also had tumors at the site of inoculation in the pectoral muscle.

The single cases which occurred in the group of chickens inoculated with the Berkefeld V filtrate and in the untreated group developed much more slowly. These were probably both spontaneous cases, since it is difficult to obtain lots of chickens for experimental use which are entirely free from this disease.

Virus Passages in Chicken Embryo Spleens.—The outgrowth from primary explants of spleens from normal 19-day-old

TABLE 1—Transmission of Avian Visceral Lymphomatosis (AVL) to Young Chickens by Intramuscular Inoculations with Supernatant Fluids from Primary Explant Tissue Cultures of Tumors from Chickens

	Inoculum					Results		
Lot No.	Origin of culture	No. medium changes	Harvest time (No. days)	Nature of inoculum	No. of chickens	AVL (%)	Muscle tumors (%)	Av. No. days to death
1	Pectoral muscle, spleen, kidney	3	13	Supernatant fluid	10	70	70	40.1
2	Pectoral muscle	2	7	Supernatant fluid	10	40	40	25.0
3	Pectoral muscle	0	6	Supernatant fluid frozen 3 months at −28 C.	10	40	10	198.8
4A	Pectoral muscle, liver, and spleen	0	2	Selas 03 filtrate of supernatant fluid	20	45	10	232.4
4B	Pectoral muscle, liver, and spleen	2	7	Berkefeld V filtrate of supernatant fluid	10	10	0	334.0
4A	Contact control	..	..	.....	5	0	0	.....
4A & 4B	Untreated controls	..	..	.....	10	10	0	316.0

chicken embryos after the migration of lymphocytes was largely fibroblastic in nature (fig. 7). The cytoplasm of the individual cells remained relatively clear. In the similar cultures to which the virus-containing supernatant fluid from chicken tumor cultures was added there was, in addition to the development of fibroblasts in the outgrowth, an extensive migration and proliferation of lymphoid tumor cells (fig. 8). The extreme granularity of the cytoplasm of cells in the virus-treated cultures, apparently similar to that described by other authors,[6,16] may indicate a cytopathogenic effect of the virus on the individual cells of the outgrowths (fig. 1, 2).

The results of chicken inoculation tests of supernatant fluids harvested from some of these virus passages in cultures of chicken embryo spleens are summarized (table 2). It appears that the virus did not survive three five-day passages. However, it apparently was still present and infective for chickens after ten three-day passages, with 1 chicken showing a lymphoid tumor in the pectoral muscle at the site of inoculation.

Birds inoculated with some of these supernatant fluids showed no resistance when challenged 38 days later with a suspension of tumor tissue containing strain RPL 12 AVL virus.

Discussion

Outgrowth from explants of neoplastic tissues is much more rapid and luxurious than from normal tissues. The migration of lymphoblasts is remarkable both in number and in speed. The character of fibroblast development seems to be about the same in both normal and neoplastic tissues.

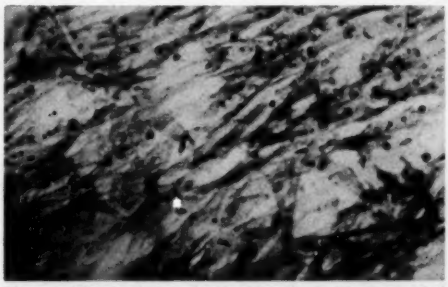


Fig. 7—Three-day culture of spleen from normal 19-day chicken embryo. Notice the predominance of fibroblasts with lymphoblasts interspersed as free wandering cells in the outgrowth. Jacobson's stain; x 174.

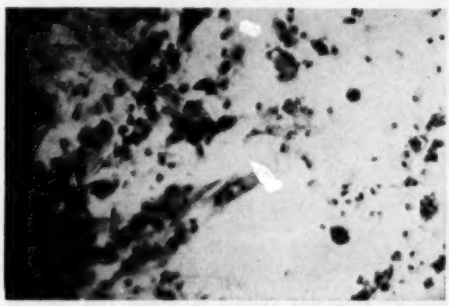


Fig. 8—Three-day culture of spleen from normal 19-day chicken embryo to which avian visceral lymphomatosis virus was added at the time of explanting. Notice the packets of lymphoblasts and single lymphoblasts which dominate the outgrowth in contrast to figure 7. Jacobson's stain; x 174.

The cultures could easily be distinguished from one another without any magnification. Cultures of neoplastic tissues were large in diameter, ragged in appearance, and the margins of the explants often were

TABLE 2—Intramuscular Inoculations of Young Chickens with Supernatant Fluids from Serial Passages of Avian Visceral Lymphomatosis (AVL) Virus on Primary Explants of Spleen from Normal 19-Day Chicken Embryos

Lot No.	Inoculum: Origin of culture	Inoculum: Challenge inoculation	No. of chickens	Results: AVL (%)	Results: Muscle tumors (%)	Results: Av. No. days to death
1	Third five-day passage		5	20	0	257.5
	Third five-day passage	Chicken tumor 38 days later	5	80	40	9.5*
2	Fifth three-day passage		5	40	0	221.0
	Fifth three-day passage	Chicken tumor 38 days later	5	100	100	6.8*
	Contact controls	.....	5	0	0	.....
3	Tenth three-day passage	.....	10	70	10	210.0
	Contact controls	.....	5	0	0	.....
4	Sixth seven-day passage	.....	10	10	0	80.0
	Untreated controls	.....	15	6.6	0	205.0

* No. of days to death after challenge.

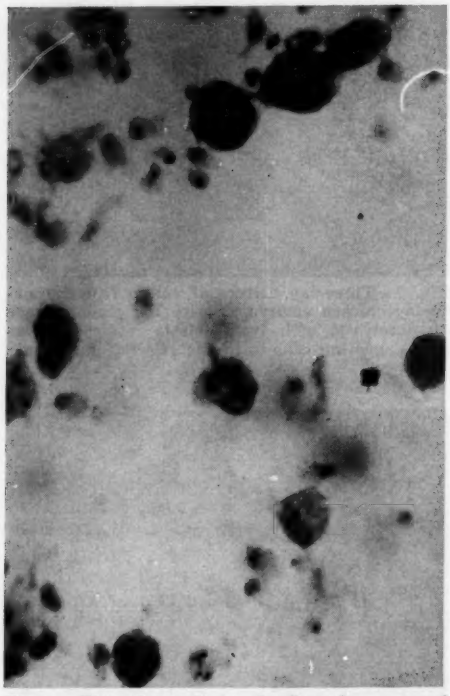


Fig. 9—Three-day culture of spleen from normal 19-day chicken embryo spleen to which avian visceral lymphomatosis virus was added at the time of explanting. Notice the packets of lymphoblasts which characterize the outgrowth. Jacobson's stain; x 656.

not clearly defined after three to five days. These characteristics were in sharp contrast to the modest appearance of the cultures of normal tissues.

The cultivation of neoplastic tissue from chickens affected with AVL *in vitro* provided useful information concerning the utility of tissue culture in the study of the disease. It should not be difficult to obtain information of the details of mitotic events in the neoplastic cells. Morphological similarities between neoplastic cells of this disease and lymphosarcomas of mammals could be more firmly established. The cells which appeared after the introduction of AVL virus in supernatant fluid from a tissue culture serial passage (fig. 9, 10) seemed indistinguishable from the description and photographs of cells of outgrowths from Hodgkin's disease cultivated *in vitro*, as shown by Murray.[19] A similar series of cells, transitional between multinucleated germinal centers and lymphoblasts and lymphocytes, was observed (fig. 11, 12).

During the first trial, explants of neoplastic liver showed only that the tissue promptly began to disintegrate in the medium, while muscle, spleen, and kidney explants showed luxurious migration and multiplication of lymphoblasts. Since the ascitic fluid in the medium had been obtained from a terminal carcinomatous patient being treated with chlorpromazine, it is felt that the chlorpromazine may have

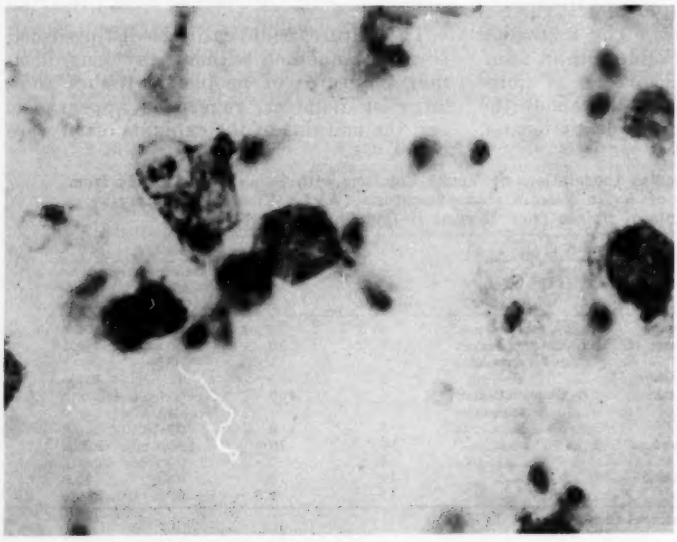


Fig. 10—Higher magnification of figure 9. Jacobson's stain; x 1017.

Fig. 11—Five-day culture of spleen from a chicken affected with avian visceral lymphomatosis. Transitional form in lymphoid series, here showing a multinucleated germinal center from which nuclei seem to migrate peripherally and break off into packets. Jacobson's stain; x 1017.

had influence on the results obtained in the culture of liver. In trial 4 when the second medium was used, outgrowth from the tumorous liver explant was as rapid and extensive as from other neoplastic tissues.

Tests of supernatant fluids of serial passages of AVL virus provided evidence that the virus had multiplied in tissue culture systems of three days' duration. Ten serial passages seemed to place the material beyond the realm of dilution or continued maintenance. Furthermore, the appearance of a tumor at the site of inoculation in the pectoral muscle was convincing evidence that the neoplasm was incited by the inoculum. Intramuscular injection of AVL virus after serial passage caused death in chickens in a length of time comparable to that commonly caused by a small dose of a suspension of frozen tumor or of a filtrate.

SUMMARY

1) Neoplastic tissues from chickens affected with avian visceral lymphomatosis (AVL) cultured *in vitro* showed marked proliferation and migration of lymphoblasts.

2) Supernatant fluids from cultures of the neoplastic chicken tissues, and Selas 03 filtrates of these fluids, produced typical AVL in a high percentage of inoculated

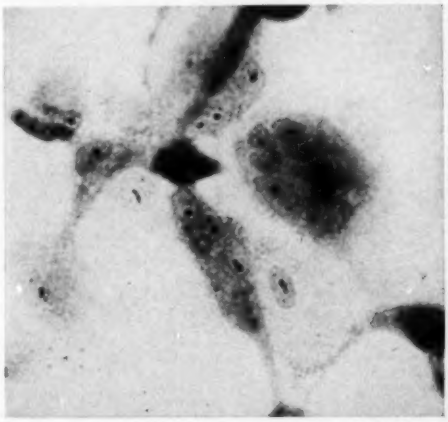


Fig. 12—Five-day culture of spleen from a chicken affected with avian visceral lymphomatosis. Transitional forms in lymphoid series showing individual cells and multinucleated forms. Jacobson's stain; x 640.

chickens. Many of these chickens showed lymphoid tumors at the site of inoculation.

3) The virus of AVL was propagated in cultures of normal chicken embryo spleens for ten three-day serial passages.

4) Packets consisting of as many as ten lymphoblasts, as well as transitional forms, were observed to characterize the cells of the outgrowth of explants of chicken embryo spleens following exposure to the virus. Fibroblasts showed marked granularity of cytoplasm.

5) Chickens which were inoculated with tissue culture material containing AVL virus but did not show evidence of neoplastic disease at 38 days were not resistant to challenge with highly virulent tumor material.

References

[1] Bichel, J.: Über eine Technik für Züchtung leukotischer Zellen *in Vitro*, sowie einige Bemerkungen über die Pathogenese der Leukosen. Arch. Zellforsch., *19*, (1940): 27–42.

[2] Carrel, A., and Burrows, M. J.: Cultivation of Sarcoma Outside the Body. J. Am. M. A., *55*, (1910): 1554.

[3] Doljanski, L., and Pikovski, M.: Cultivation of the Agent of Fowl Leukosis *in Vitro*. Nature (London), *146*, (1940): 302–303.

[4] Doljanski, L., and Pikovski, M.: Cultures *in Vitro* of Blood Cells, Bone Marrow, and Myocardium from Leukotic Fowls. Cancer Res., *1*, (1941): 205–216.

[5] Doljanski, L., and Pikovski, M.: Agent of Fowl Leukosis in Tissue Cultures. Cancer Res., *2*, (1942): 626–631.

[6] Fontes, A. K., Burmester, B. R., and Isler, P. E.: Cultivation in Tissue Culture of the Virus of Avian Visceral Lymphomatosis. Proc. 46th Ann. Meeting Poult. Sci. A., (1957): 17–18.

[7] Furth, J., and Breedis, C.: Attempts at Cultivation of Viruses Producing Leukosis in Fowls. Arch. Path., *24*, (1937): 281–302.

[8] Furth, J., and Stubbs, E. L.: Tissue Culture Studies on the Relation of Sarcoma to Leukosis of Chickens. Proc. Soc. Exptl. Biol. & Med., *32*, (1934): 381–383.

[9] Gustafson, D. P., and Pomerat, C. M.: Cytopathic Changes in Tissue Cultures Derived from a Pig Infected with Hog Cholera. Am. J. Vet. Res., *17*, (Jan., 1956): 165–173.

[10] Ludford, R. J.: The Production of Tumours by Cultures of Normal Cells Treated with Filtrates of Filterable Fowl Tumours. Am. J. Canc., *31*, (1937): 414–429.

[11] Murray, Margaret R.: The Classification and Diagnosis of Human Tumors by Tissue Culture Methods. Texas Rep. Biol. & Med., *12*, (1954): 898–915.

[12] Ruffilli, D.: Su Alcuni Caratteridi un Ceppo di Cellule Mesenchimali Ottenute da Milza di Pollo Eritroleucemico. Boll. Lega ital. Tumori, *10*, (1936): 223–225.

[13] Ruffilli, D.: Coltivazione di Tessuti di Polli Eritroleucemico. Boll. Lega ital. Tumori, *11*, (1937): 169–172.

[14] Ruffilli, D.: Azione del Plasm di Pollo Eritroleucemico su Tessuti Coltivati *in Vitro*. Boll. Lega ital. Tumori, *12*, (1938): 235–246.

[15] Ruffilli, D.: Infezioni *in Vitro* di Esplanti di Cuore Embrionale con Cellule del Sangue di Pollo Eritroleucemico. Boll. Soc. ital. Biol. sper., *13*, (1938): 146–148.

[16] Ruffilli, D.: Considerazioni su Recente Riccerche sulla Coltivazione *in Vitro* di Virus Aviari. Rass. Oncol., *16*, (1938): 32–41.

[17] Sharpless, G. R., Defendi, V., and Cox, H. R.: Cultivation in Tissue Culture of the Virus of Avian Lymphomatosis. Proc. 46th Ann. Meeting Poult. Sci. A., (1957): 56.

[18] Van den Berghe, L., and d'Ursel, F.: Culture en Tissus du Virus de l'Erythroblastose de la Poule (souche O. G.). Comp. rend. Soc. biol., *131*, (1939): 1301–1302.

[19] Verne, J., Oberling, C., and Guerin, M.: Tentatives de Culture *in Vitro* de l'Agent de la Leucémie Transmissible des Poules. Compt. rend. Soc. biol., *121*, (1936): 403–405.

Swine Kidney Cell Cultures—Susceptibility to Viruses and Use in Isolation of Enteric Viruses of Swine

B. B. HANCOCK, D.V.M., M.S.; E. H. BOHL, D.V.M., Ph.D.;
J. M. BIRKELAND, Ph.D.

Columbus, Ohio

THE REFINEMENT and application of tissue culture techniques have resulted in the isolation of many viruses which previously were unrecognized. The recent discovery and characterization of numerous viral agents from the intestinal tract of man lend support to the hypothesis that similar agents are present in the intestinal tracts of animals and are of possible significance in intestinal and, perhaps, other disorders either as primary or secondary pathogens.

Since swine often suffer from gastrointestinal disturbances in which the specific etiological agent is not always discernible, an investigation of enteric viruses of swine was undertaken. It was the purpose of this study to (1) explore and develop tissue culture systems of swine kidney cells, (2) determine the susceptibility of such cells to known viruses, and (3) determine the value of such a system for the isolation of viruses from the intestinal tracts of swine.

The literature on the cultivation of swine kidney cells and their use in virus studies is limited. Millian and Robbins[10] investigated the nutritional requirements for the cultivation of porcine kidney epithelial cells, using fragments planted by a dry tube or plasma clot technique. Satisfactory growth was obtained in a medium composed of synthetic mixture 199 and 7.5 per cent porcine serum.

Lee[6] obtained good growth of epithelial cells from trypsinized porcine kidney tissue in a medium consisting of 0.5 per cent lactalbumin hydrolysate and 10.0 per cent equine serum in balanced salt solution. Using the conglutinating complement-absorption test, he demonstrated the proliferation of the transmissible gastroenteritis virus of swine, although no cytopathogenic effects were observed.

Fieldsteel and Emery[2] cultivated cells from porcine kidney tissue in a lactalbumin hydrolysate medium containing equine serum and reported that no cytopathogenic changes occurred after inoculation with infectious canine hepatitis virus.

Moscovici *et al.*[11] have reported the isolation of a cytopathogenic agent from an outbreak of enteritis in newborn pigs in Italy.

Beran *et al.*,[1] using tissue culture methods, isolated a number of cytopathogenic agents from the feces of pigs, but their investigation indicated that the isolates belong to a single type.

Young *et al.*[15] reviewed the literature on the cultivation of swine cells and reported the cultivation of swine kidney cells in static cultures, using a variety of nutrient solutions.

Recently, Madin *et al.*[9] have described the preparation of tissue cultures from a wide range of animal tissues, including swine kidney tissue.

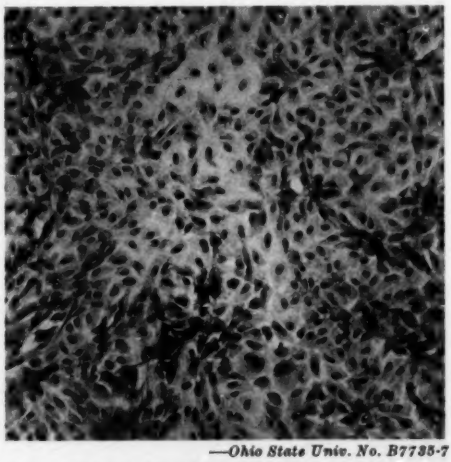
While this manuscript was in preparation, Guerin and Guerin[5] reported on the susceptibility of a pig kidney tissue culture to a number of viruses found in man.

EXPERIMENTAL PROCEDURE AND RESULTS

Equipment Preparation.—Culture tubes were standard 16- by 150-mm. Pyrex test tubes, or those with the lower one third of the tube flattened (Leighton tubes). In some instances, ordinary prescription bottles were used.

All used glassware was immediately immersed in "7x" detergent* solution diluted 1:10. After

* Produced by the Limbro Chemical Co., New Haven, Conn.



—Ohio State Univ. No. B7735-7

Fig. 1—Normal monolayer cell culture of adult swine kidney. May-Grünwald-Giemsa stain; x 80.

From the Department of Bacteriology, Ohio State University, Columbus.

This investigation was supported in part by Grant E-1352 from the National Institutes of Health, U.S. Public Health Service, a grant from Fort Dodge Laboratories, Inc., and the Ohio Agricultural Experiment Station.

XUM

TABLE 1—Viruses Tested in Swine Kidney Tissue Cultures

Virus	Origin	Titer*	Titer†
Herpes simplex	HeLa cell culture	10^{-1}	$10^{-2.3}$
Pseudorabies	Mouse brain	$10^{-2.5}$	$10^{-4.5}$
Infectious canine hepatitis	Dog kidney tissue culture	$10^{-3.5}$	$10^{-4.3}$
Enteric cytopathogenic human orphan (strain 10)	Monkey kidney tissue culture	$10^{-3.7}$	$10^{-4.5}$
Newcastle disease	Chicken embryo	$10^{-5.3}$	$10^{-5.3}$

* Fifth tissue culture passage; † tenth tissue culture passage.

soaking overnight or longer, it was brushed and then rinsed with an automatic washer† (5 minutes with tap water, 3 minutes with distilled water). A manual rinse in deionized water completed the procedure.

White, nontoxic rubber stoppers were washed by soaking in the detergent and rinsed by vigorous tumbling in running water, first tap and then distilled. They were sterilized by boiling in deionized water and transferred directly to the culture tubes with sterile forceps.

Tissue Culture Mediums and Reagents.—Hanks' balanced salt solution was prepared according to the method of Weller *et al.*[14] It was sterilized by autoclaving at 10 lb. of pressure for ten minutes, and stored at 5 C. in tightly stoppered prescription bottles. The pH was adjusted immediately before use with sodium bicarbonate solution.

A modified lactalbumin hydrolysate** medium was the basic medium used in this work. A 0.5 per cent solution of lactalbumin hydrolysate was prepared in Hanks' solution, distributed in prescription bottles, autoclaved at 10 lb. of pressure for ten minutes, and stored at 5 C. Serum and antibiotics were added and the pH was adjusted with 4.4 per cent sodium bicarbonate solution just before use. Porcine serum (3.0–10.0%) was used for the growth medium, and bovine serum (3.0%) for the maintenance medium. These mediums were selected on the basis of comparative studies of various mediums for the growth and maintenance of swine kidney cells.[6]

Swine and bovine serums were collected from young, healthy animals at slaughter, sterilized by filtration through Selas 03 or Seitz filters, and stored frozen in convenient amounts.

The trypsin solution was a 0.25 per cent solution of Bacto-Trypsin 1:250 (Difco) in Hanks' solution. Sodium bicarbonate was prepared as a 4.4 per cent solution in deionized water and sterilized by filtration. Antibiotics, enough to yield a final concentration of 50 units of nystatin (Mycostatin‡), 100 μg. of streptomycin, and 200 units of penicillin per milliliter, were added to all tissue culture fluids.

Preparation of Cultures.—Kidneys were aseptically removed from 6- to 8-month-old swine, slaughtered at a nearby abattoir, and brought to the laboratory in a sterile, covered pan. After removing the capsule, portions of the cortex were transferred to a 50-ml. centrifuge tube, minced to 3- to 5-cmm. fragments with long-handled scissors, and washed free of erythrocytes and debris with trypsin solution containing antibiotics.

The washed fragments were transferred to an Erlenmeyer flask and trypsin solution, prewarmed to 37 C., was added. The suspension was agitated with a magnetic mixer at a speed which maintained vigorous agitation without foaming. To prevent heating, the flask was suspended 0.5 inch above the mixer. After a preliminary trypsinization for one to two hours at room temperature (approx. 25 C.) the supernatant fluid was discarded and the fragments were washed several times with trypsin. Three volumes of trypsin solution then was added to the flask and agitation was continued for another hour. After straining through one or two layers of gauze, the resulting cell suspension was centrifuged at 600 r.p.m. for five minutes in an International No. 1 centrifuge. The cells were washed one or two times with complete growth medium at 37 C. and centrifuged each time at 600 r.p.m. for two or three minutes.

An indication of the viability of the trypsinized cells was obtained by staining with trypan blue, using a technique modified from that described by Girardi *et al.*[4] A wire loop was used to transfer equal amounts of cell suspension and stain to a clean slide. A cover slip was added and the preparation examined under the microscope. A similar technique, employing 0.1 per cent crystal violet in 0.1 M citric acid, was used to study the morphological characteristics of the cells.

The final suspension usually represented a 1:200 dilution of the packed cell volume in the complete growth medium, although variations of this concentration were used in some experiments. Distribution of the suspension was done by a Cornwall automatic syringe. Tubes usually received 1.5 to 2.0 ml., and 4-oz. prescription bottles were planted with 10.0 ml. The tubes and bottles were stoppered tightly and incubated at 37 C. in a stationary position.

In most lots of cultures, definite colonies had developed after 48 hours' incubation. Multiplication then proceeded more rapidly and the entire growth surface was covered in six to ten days (fig. 1). One or two changes of medium were necessary during this period.

Several attempts have been made to propagate serially the swine kidney epithelial cells. Good growth was obtained on the first passage and, in one experiment, the passage cells appeared as susceptible to herpes simplex virus as were the original cells. In the most successful attempt, it was possible to carry the cells into the fourth passage. At

† Manufactured by Heinicke Instruments, Hollywood, Fla.

** Produced by Nutritional Biochemicals Corp., Cleveland, Ohio.

‡ Mycostatin is produced by E. R. Squibb and Sons, New York, N.Y.

that time, they were growing slowly and it was impossible to obtain a confluent sheet of cells.

Effect of Certain Viruses on Swine Kidney Cell Cultures.—The herpes simplex virus was obtained from infected HeLa cell cultures;* the pseudorabies virus was from brain emulsion of infected mice; infectious canine hepatitis virus was from the sixteenth passage in canine kidney tissue cultures;† the enteric cytopathogenic human orphan virus 10 (ECHO-10) was from infected monkey kidney tissue cultures;** and the Newcastle disease virus was from the allantoic fluid of infected chicken embryos.

Before inoculation, the medium was replaced with lactalbumin hydrolysate medium containing 3 per cent porcine serum. Inoculation was performed by adding 0.1 ml. of the virus preparation directly to each of three or more cell cultures. Cultures were observed daily and subcultures made when extensive cytopathogenic changes were evident. Each virus was carried through ten passages and titrated at the fifth and tenth passages.

Titrations were made by preparing tenfold dilutions in Hanks' solution and inoculating three tubes with 0.1 ml. of each dilution. End points were calculated as the tissue culture infective dose ($t.c.i.d._{50}$) according to the method of Reed and Muench.

A summary of the results of inoculation of the swine kidney cells with the viruses used is given (table 1).

* Obtained from Dr. George Anderson, † Dr. Victor Cabasso, and ** Dr. W. MsD. Hammon.

Herpes simplex virus produced a cytopathogenic effect on swine kidney cells that was characterized by the production of rounded, highly refractile cells which soon lost their attachment to the glass. The result was a steadily enlarging, denuded area surrounded by reactive cells. On continued passage, the cytopathogenic effect first was diminished and delayed, but the eighth, ninth, and tenth passages resulted in increased cytopathogenicity.

Pseudorabies virus produced a cytopathogenic effect on siwne kidney cells which was similar to that produced by herpes simplex, but more dramatic. The cells became markedly swollen and rounded, and tended to separate.

Infectious canine hepatitis virus produced a cytopathogenic effect which differed from that of pseudorabies virus in two respects. In general, the corresponding cellular changes occurred approximately 24 hours later (2 to 3 days) and fewer swollen cells were observed.

The ECHO-10 virus produced a cellular destruction which was complete in five to six days. The only specific pathological change observable in unstained preparations was the destruction of the cytoplasm and pyknosis of the nuclei. The affected

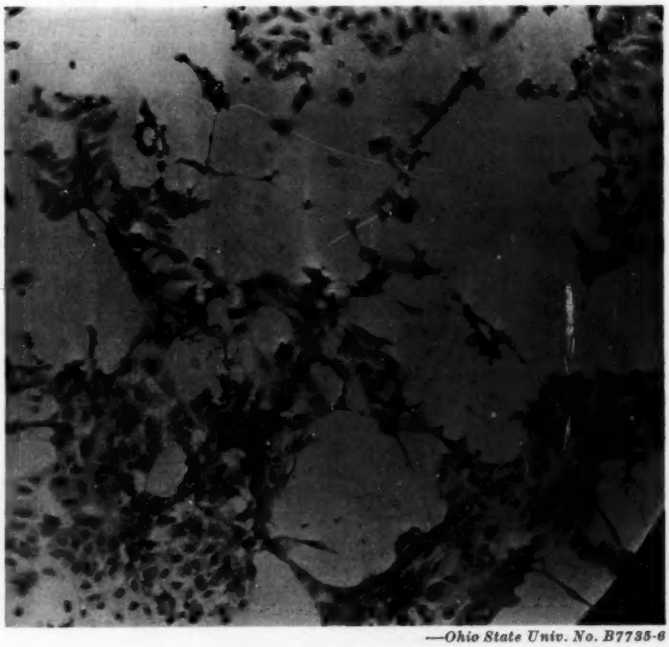


Fig. 2—Swine kidney cells inoculated with unknown cytopathogenic agent from swine. May-Grünwald-Giemsa stain; x 80.

—Ohio State Univ. No. B7735-6

XUM

cells rapidly lost their attachment to the glass surface.

Newcastle disease virus caused a generalized destruction of the swine kidney cells. The first change, observable at 12 to 24 hours, was a granulation of the cytoplasm. Usually, significant numbers of cells were destroyed by 24 hours, and the destruction then proceeded at a more rapid rate.

Virus Isolations From Swine.—Rectal swab samples were collected from pigs by a technique similar to that used on children by Ramos-Alvarez and Sabin.[12] The swabs were extracted with 1.0 to 2.0 ml. of Hanks' solution containing 1,000 units of penicillin, 500 μg. of streptomycin, and 250 units of nystatin per milliliter. The resulting suspension was centrifuged at approximately 4,000 r.p.m. at room temperature for 30 to 40 minutes in an angle centrifuge. Tissue culture tubes were then inoculated with 0.1 to 0.2 ml. of the supernatant fluid.

Fecal samples were prepared for inoculation by grinding, in a mortar, a 20 per cent suspension of feces in Hanks' solution. Antibiotics were added to give a final concentration of 1,000 μg. of streptomycin, 500 units of nystatin, and 2,000 units of penicillin per milliliter. The suspension then was centrifuged and the supernatant fluid inoculated as described. Before inoculation, the kidney cell cultures were washed with Hanks' solution and lactalbumin hydrolysate medium containing 3 per cent bovine serum was added as a maintenance medium.

Cells were examined at least every two days and usually every day for evidence of cytopathogenic effect. In case of direct cytotoxicity of the fecal suspensions, which occurred often, the fluids were harvested on the third to fifth day; otherwise, they were harvested at seven to ten days. When definite cytopathogenic effects were observed, harvests were made after extensive destruction of the cells was evident. At least one blind passage was made before samples were considered negative.

Several cytopathogenic agents have been isolated from the intestinal tracts of both normal swine and those showing enteric disturbances. In each case, one isolate from a given group of animals was carried through 15 passages in swine kidney cell cultures, including several titrations. Filtration experiments conducted on each showed that all passed a Selas 03 filter with little or no loss of titer. Bacteriological examinations of the culture fluid, using standard mediums for aerobic and anaerobic bacteria, fungi, and pleuropneumonia-like organisms were negative.

The following is a brief account of the isolation of these agents and of the cytopathogenic changes produced in swine kidney cultures inoculated with 100 $t.c.i.d._{50}$ of the later passages.

Rectal swab samples were collected from a group of 8-week-old pigs with severe diarrhea. Of the nine samples tested, only one (virus 11-45) continued to cause destruction of the cells when subcultured. In cultures inoculated with virus 11-45, degeneration was first observable at approximately 36 hours, after which the cells were rapidly destroyed and fell from the glass surface. Destruction was complete within 72 hours. Attempts to isolate this agent from the same animal several weeks later were unsuccessful.

Virus 11-56b was isolated from an animal in the same herd as the animal from which virus 11-45 was isolated, approximately ten weeks later, at which time none of the pigs had diarrhea. This agent produced a type of cytopathogenic change entirely different from that caused by other agents currently under study. Rounded, swollen, highly refractile cells were observed within 24 hours after inoculation, and complete destruction occurred within 72 hours.

Virus 6-6 was isolated from rectal swabs collected from a drove of shoats with an enteric disturbance. Almost all swine brought onto this farm over the past several years have developed diarrhea a few days after arrival. The clinical disease is mild and runs its course in a few days. Cytopathogenic changes were observed in the second passage of all samples and sample 6-6 was selected for further study. This agent causes rapid destruction of the swine kidney cells, which is first observed at 24 hours (fig. 2) and is usually complete at 48 hours.

Virus H2 was isolated from fecal samples of an apparently normal pig. Cytopathogenic changes were observed in the cultures inoculated with the original sample.

Preliminary studies on the characteristics of these viruses have been conducted and are summarized (table 2).

Inoculation of 21-day-old Swiss mice with these agents was by intracerebral, intraperitoneal, and subcutaneous injection. Rabbits were inoculated subcutaneously and intravenously, in attempts to produce neutralizing antiserums. Monkey kidney cell cultures* and bovine kidney cultures prepared in the same way as the swine kidney cultures were inoculated with 0.1 ml. of tissue culture fluid.

Difficulty has been experienced in producing satisfactory neutralizing antiserums.

* Obtained from Microbiological Associates, Washington, D.C.

XUM

TABLE 2—Characteristics of Viruses Isolated from Swine

Virus	Visible effects in: Porcine kidney t.c.	Bovine kidney t.c.	Monkey kidney t.c.	Swiss mice	Rabbits	Inactivated at 65 C. in 30 min.	Av. titer
11-45	+	−	+	−	−	+	$10^{-5.8}$
11-56b	+	−	−	−	−	−	$10^{-5.5}$
6-6	+	−	+	−	−	−	$10^{-5.3}$
H2	+	−	+	−	−	+	$10^{-5.0}$

t.c. = tissue culture.

Rabbits injected intramuscularly with large doses of live virus in mineral oil adjuvant and then given live virus intravenously at weekly intervals produced only low-titer (1:64) neutralizing antiserums. Attempts to produce antiserums in mice, chickens, and guinea pigs have been unsuccessful.

In addition to the agents listed above, several additional cytopathogenic agents have been isolated from the intestinal tracts of swine. In all, approximately 30 isolations have been made from eight different herds.

Discussion

A procedure for the preparation of cultures of swine kidney cells has been developed, with emphasis given to the simplification of the technique. Confluent sheets of epithelial-type cells in tubes and in bottles have been obtained consistently.

The cytopathogenicity of several viruses for these cells has been studied. Some of these results warrant further consideration. The steadily diminishing cytopathogenicity observed on passages of herpes simplex virus and the rapid cytopathogenicity of pseudorabies virus is in direct contrast to results reported for these viruses in the HeLa cell.[13] While it is not possible to eliminate the possibility of rapid inactivation of herpes simplex virus, there seemed to be a decrease in cytopathogenicity for the swine kidney cells, followed by an increase on further passage. Such an explanation would be consistent with considerations of the natural hosts of these two viruses and the origins of the cells involved. The swine kidney cells afford a valuable system for the study of the relationships of these viruses.

Our results with infectious canine hepatitis virus were not in agreement with those of Fieldsteel and Emery.[2] However, the strain used in our experiments was of tissue culture origin. Gillespie[3] recently has reported the growth and cytopathogenicity of a tissue culture-adapted virus in swine kidney cells.

The ECHO-10 virus also was found by Guerin and Guerin[5] to be cytopathogenic for swine kidney cells, although other members of the ECHO group are not. Limited trials in our laboratory with other ECHO viruses have given similar results. The ECHO-10 virus is an unusual member of this group, in that it has been adapted to suckling mice by repeated blind passage.

A number of cytopathogenic agents have been isolated from the intestinal tracts of swine. That these agents should be considered viruses is indicated by their propagation in the swine kidney cultures, filterability, resistance to antibiotics, and failure to grow on inert mediums. The relative ease with which the agents were recovered indicates that such viruses are widespread. The techniques were such as to select only the more resistant viruses that might be present and those that would produce a rapid destruction of the swine kidney cells. Preliminary studies indicate that these viruses, as a group, are resistant to physical and chemical agents.

The significance of these isolated agents can not be assessed at this time. It was not one of the objectives of this preliminary study to do so; it merely confirmed the expected. Huebner[7] stated: "New techniques . . . have reduced the isolation of new human and animal viruses from a technological feat of high order to an almost exasperatingly commonplace occurrence."

The apparent prevalence of such agents leads directly to the problem of nomenclature or specific designation. If the system applied to other similar enteric viruses is to be followed, it seems logical that these agents should be designated as "enteric cytopathogenic porcine orphan" (ECPO) viruses and numbers assigned to each strain. It would be necessary to show first that they are antigenically distinct from such viruses isolated from other animals.

Veterinary medicine has two major advantages in the study of such orphan viruses. It is possible to perform infectivity studies in the host animal and, if necessary,

XUM

to kill the animal to study fully the effects of the disease. This is particularly important with those agents which rarely produce a fatal illness to provide necropsy material. A second major advantage is the ready-made, limited, and confined populations which are available for study. Such studies are in progress.

SUMMARY

1) A simple technique has been used to culture successfully, *in vitro*, epithelial-type cells from porcine kidneys obtained at abattoirs.

2) The following known viruses have been found to produce cytopathogenic effects in these cells: herpes simplex, pseudorabies, infectious canine hepatitis, Newcastle disease, and enteric cytopathogenic human orphan (strain 10).

3) Viruses were isolated readily from the intestinal tracts of swine, using the described tissue culture system. So far, 30 unidentified viruses from eight swine herds have been isolated. Attempts are being made to determine their antigenic relationships and their role, if any, in disease production. Characteristics of four of these agents are presented.

References

[1] Beran, G. W., Werder, A. A., and Wenner, H. A.: Enteroviruses of Swine. Presented at 85th Ann. Meet. Am. Pub. Health A., Nov., 1957.

[2] Fieldsteel, A. H., and Emery, J. B.: Cultivation and Modification of Infectious Canine Hepatitis Virus in Roller Tube Cultures of Dog Kidney. Proc. Soc. Expti. Biol. & Med., *86*, (1954): 819–823.

[3] Gillespie, J. H.: Infectious Canine Hepatitis. J.A.V.M.A., *132*, (Jan. 1, 1958): 1–2.

[4] Girardi, A. J., McMichael, H., Jr., and Henle, W.: The Use of HeLa Cells in Suspension for the Quantitative Study of Virus Production. Virology, *2*, (1956): 532–544.

[5] Guerin, L. F., and Guerin, M. M.: Susceptibility of Pig Kidney Tissue Cultures to Certain Viruses. Proc. Soc. Exptl. Biol. & Med., *96*, (1957): 322–323.

[6] Hancock, B. B.: The Cultivation of Swine Kidney Cells and Their Use in Virus Studies. M.S. thesis, Ohio State University, Columbus, 1957.

[7] Huebner, R. J.: The Virologists' Dilemma. Ann. New York Acad. Sci., *67*, (1957): 430.

[8] Lee, K. M.: Propagation of Transmissible Gastroenteritis Virus in Tissue Culture. Ann. New York Acad. Sci., *66*, (1956): 191–195.

[9] Madin, S. H., Andriese, B. S., and Darby, N. B.: The *in Vitro* Cultivation of Tissues of Domestic and Laboratory Animals. Am. J. Vet. Res., *18*, (Oct., 1957): 932–941.

[10] Millian, S. J., and Robbins, K. C.: *In Vitro* Cultivation of Porcine Kidney. Proc. Soc. Exptl. Biol. & Med., *91*, (1956): 548–551.

[11] Moscovici, C., Ginevri, A., and Mazzaracchio, V.: Isolamenta su Coltura di Tissuti di un Virus da Enterite Suina. Zooprofilassi, *1*, (1956): 417–426.

[12] Ramos-Alvarez, M., and Sabin, A. B.: Intestinal Flora of Healthy Children Demonstrable by Monkey Kidney Tissue Culture. Am. J. Pub. Health, *46*, (1956): 295–299.

[13] Scherer, W. F., and Syverton, J. T.: The Viral Range *in Vitro* of a Malignant Human Epithelial Cell (Strain HeLa-Gey) I. Multiplication of Herpes Simplex, Pseudorabies, and Vaccinia Viruses. Am. J. Path., *30*, (1954): 1057–1073.

[14] Weller, J. H., Enders, J. F., Robbins, F. C., and Stoddard, M. D.: Studies on the Cultivation of Poliomyelitis Viruses in Tissue Culture. I. The Propagation of Poliomyelitis Viruses in Suspended Cell Cultures of Various Human Tissues. J. Immunol., *69*, (1952): 645–671.

[15] Young, G. A., Underdahl, N. R., and Sabina, L. R.: Swine Tissue Culture Systems. I. Trypsin-Dispersed Kidney. Am. J. Vet. Res., *18*, (July, 1957): 466–472.

Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus
I. Propagation and Pathogenicity

RAYMUNDO G. CUNHA, D.V.M., and ERVIN A. EICHHORN, V.M.D.

Rio de Janeiro, Brazil

THE SUSCEPTIBILITY of rabbits to foot-and-mouth disease virus was described many years ago by several authors.[1, 2, 13, 25]

Inoculation of the tongue of the rabbit results in the formation of a primary vesicle and transmission in series can be obtained successfully, but the development of secondary lesions is not of regular occurrence and is limited to the buccal mucous membranes.

The recognition of the greater suceptibility of the guinea pig led to the abandonment of work with rabbits.

Skinner's publication,[22] describing the susceptibility of unweaned white mice to foot-and-mouth disease virus, opened the way to a series of new investigations on the susceptibility of newborn animals. Infection subsequently has been produced in newborn rats, hamsters, pups, calves, kids, lambs, and colts, and in day-old chicks.[11, 12, 14, 15, 21, 22, 24]

Nagel,[15] in reporting the susceptibility of suckling guinea pigs, pigs, kittens, and rabbits, described the production of two successive passages of foot-and-mouth disease virus in rabbits.

Gribanov [16] described the use of a vaccine prepared from virus propagated in rabbits.

Verge *et al.*[23] recently reported the successful serial passage of strains of three types of foot-and-mouth disease virus in rabbits less than 9 days old.

In April, 1952, prior to Nagel's publication, the authors began to investigate the propagation of foot-and-mouth disease virus in suckling rabbits, in the hope of developing a new source of virus for vaccine production. This species was selected because the gestation period and the rate of fetal development are similar to those of mice.

Reference has been made to preliminary results in various publications; a report on the histological lesions in rabbits has been published.[4, 9, 10, 19]

In this paper, a description is given of the propagation in suckling rabbits of strains of foot-and-mouth disease virus of the Vallée O, Vallée A, and Waldmann C types. The distribution of the virus in the rabbit and the results of the inoculation of cattle with the virus passaged in rabbits also are reported.

MATERIALS AND METHODS

Strains of Foot-and-Mouth Disease Virus.—Strains of Vallée O, Vallée A, and Waldmann C types, that had originated from natural outbreaks of foot-and-mouth disease in cattle in the state of Rio de Janeiro, Brazil, were used. These have been identified as the Nilopolis, São Bento, and Nova Iguaçu strains, respectively.

The type O strain had been passaged once and the type A strain had been passaged five times in unweaned mice before being used for rabbit inoculation. The type C strain was used without previous passage.

Rabbits.—The rabbits used were purchased from a commercial breeder; mothers and their young were received 24 hours after birth of the litters. Rabbits of increasing age were used in the course of the experiments; those 50 days old or older had been weaned previously.

Rabbit Inoculation and Passages.—All infective tissues were ground with sterile sand and suspended in sterile phosphate buffer solution at a pH of 7.5 to 7.7 to form a 1:4 suspension. This suspension was clarified by centrifugation and the supernatant fluid was mixed with an equal volume of a mixture of penicillin and streptomycin so as to contain 1,000 units of penicillin and 1 mg. of streptomycin per milliliter in the final 1:9 suspension.

The suspension was stored at 4 C. for 30 minutes. Before inoculation, a portion was added to fluid thioglycollate medium, in a test of sterility. Filtrates of the muscle suspensions of passages 20, 78, 86, and 136 of the O type strain and of the twentieth passages of the A and C type strains were prepared, using pads of Seitz EK porosity.

A group of 3 to 8 rabbits was selected for each passage, but 1 to 3 were left uninoculated to serve as specificity controls. The rabbits were injected intraperitoneally with 0.25 to 1.0 ml. of the suspension, according to age.

The rabbits were observed several times a day for two weeks after inoculation. All sick animals were observed at 10:00 p.m., and if found *in extremis* they were transferred to a cold room (4 C.). Dead rabbits were stored at −20 C. The tissue usually harvested was leg muscle but, if a large amount of virulent material was required, all the musculature was used. The harvested tissue was stored at −20 C.

On a few occasions, the heart, lungs, liver,

From the Pan American Foot-and-Mouth Disease Center of the Pan American Sanitary Bureau, Rio de Janeiro, D.F., Brazil. Dr. Eichhorn is presently with the FAO, Rome, Italy.

The authors thank Dr. W. M. Henderson, director of the Center, for his criticism and review of the text; Mr. Oziel Oliveira, for his help with the inoculation and observation of the experimental animals during the latter part of the investigation; and Dr. J. L. Saile, for performing the majority of the complement-fixation tests.

spleen, kidneys, and brain also were harvested.

Titration of Virus Suspensions in Unweaned Mice.—Tenfold serial dilutions were prepared, using phosphate buffer. Each dilution was inoculated intraperitoneally into groups of 4 to 8 unweaned mice, 6 to 8 days old, using 0.05 ml. per mouse. For titration of muscle tissue, not less than 3 Gm. was taken, from several muscles. The titration of other tissues was performed by using a pool of all identical tissues of the same passage. Fifty per cent end points were calculated by the method of Reed and Muench.[20]

Cattle.—The cattle used were 14 to 18 months old, and were obtained from various farms in the state of Rio de Janeiro. They were crossbred, with Zebu characteristics predominating.

Cattle Inoculation.—The virus was inoculated intradermally into the tongue, using approximately 0.1 ml. of inoculum at each site. Virus titrations in cattle were performed by Henderson's method,[17] using chloral hydrate anesthesia.

Unless stated otherwise, the cattle were selected on the basis of previous examination of their serums for the absence of foot-and-mouth disease antibodies, using a serum-protection test in unweaned mice.[5]

Complement-Fixation Test.—A technique employing 50 per cent complement units and spectrophotometric readings was used.[3,7]

RESULTS

Propagation of Virus in Rabbits—Strain Nilopolis, Vallée O Type.—This strain has been propagated through 137 passages. The first 42 passages were made in 1- to 2-day-old rabbits.

By the fortieth passage, it was found that the virus still was pathogenic for cattle. Unsatisfactory results were obtained with a formolized aluminium hydroxide vaccine prepared from this virus. It then was thought worthwhile to investigate the possibility of adapting the virus to older rabbits by gradually increasing the age of the rabbits used during the series of passages (table 1).

From the first to the forty-ninth passage, muscle was titrated in unweaned mice each third or fourth passage. From the forty-ninth passage on, as more facilities became available, muscle from each passage was titrated.

TABLE 1—Age of Rabbits Used in Passage of the O Type Strain of Foot-and-Mouth Disease Virus

Passage No.	Age of rabbits (days)
1 to 42	1 to 2
43 to 54	8 to 10
55 to 69	14 to 16
70 to 77	18
78 to 94	21
95 to 98	24
99 to 103	28
104 to 107	30
108 to 111	40
112 to 137	50 to 51

Muscle from the first passage had a titer of $10^{3.3}$ but, from the fifth to the twentieth passages, the titers ranged from $10^{4.5}$ to $10^{6.6}$. With a further increase in the number of passages to the forty-second, still higher titers (10^{7} to $10^{8.3}$) were obtained. After this, older rabbits were used, and it was observed that whenever the age of the rabbits was increased the titers tended to drop for the first few passages and then rise again to a level of about $10^{7.5}$.

Strain São Bento, Vallée A Type.—A total of 40 passages was made with this strain, using 1- to 15-day-old rabbits. For passages 1 to 22, the rabbits were 1 to 7 days old; for passages 23 to 30, they were 7 to 8 days old; and for passages 31 to 40, they were 14 to 15 days old.

Infected muscle was titrated from passages 2, 5, 15, 20, and all subsequent passages.

The titers of virus ranged from $10^{3.5}$ to $10^{7.5}$. As with the O type strain, the titer increased with the number of passages in rabbits of the same age.

Strain Nova Iguaçu, Waldmann C Type.—Forty passages were completed with the C type strain. One- to 2-day-old rabbits were used for passages 1 to 24, 7- to 8-day-old rabbits for passages 25 to 28, and 14- to 15-day-old rabbits for passages 29 to 40.

Muscle was titrated from passages 5, 20, 23, and all subsequent passages. The titers ranged from 10^{4} to $10^{7.5}$.

Complement-Fixation Control.—Throughout the passages, antigens were prepared from infected rabbit muscle and checked by the complement-fixation test. The results of these tests have shown that, with each strain, the virus type has remained unchanged.

Distribution of Virus in Different Rabbit Tissues.—At four different passages of the O type strain and at one each of the A and C type strains, tissues other than muscle were harvested for titration in unweaned mice. The tissues or organs were harvested separately, washed lightly in normal saline solution, and pooled according to the number of rabbits used for the passage.

With the O type strain, titrations were made of various tissues from passages 16, 40, 50, and 76, for which 7, 4, 3, and 3

TABLE 2—The Distribution of Foot-and-Mouth Disease Virus in Various Tissues of Rabbits of Different Ages

Passage No.	Virus type	Age of rabbits (days)	Virus titers determined in suckling mice						
			Muscle	Heart	Lung	Liver	Spleen	Kidney	Brain
16	O	2	5.6	3.5	3.5	3.5	.....	3.5	3.4
40	O	2	7.0	6.0	4.5	5.3	4.6	4.5	3.7
50	O	1	8.0	6.8	6.3	6.3	7.2	6.0	4.8
76	O	18	7.3	4.6	4.3	4.3	4.3	4.6	.....
20	A	5	5.7	4.5	4.5	4.5	5.0	4.7	3.3
20	C	4	6.5	5.0	5.8	5.2	4.3	4.8	3.7

Titers expressed as powers of 10.

rabbits, respectively, had been used. Titrations, using tissues of the twentieth passages of the A and C type strains were made; 4 rabbits had been used in each case (table 2).

In all instances, muscle tissue showed the highest titer and brain tissue the lowest.

Attempts to Infect Older Rabbits with the O Type Strain.—Unsuccessful attempts were made on several occasions to infect rabbits considerably older than those being used.

Virus of the second passage infected rabbits 2 days old, but not those 4 days old. The sixty-fifth passage infected 15-day-old rabbits, but not 32-day-old rabbits. The seventy-fourth passage infected 18-day-old but not 31-day-old rabbits. However, rabbits 24 days old were being used successfully by the ninety-fifth passage, 28 days old by the ninety-ninth passage, 30 days old by the 104th passage, 40 days old by the 108th passage, and 50 and 51 days old by the 112th and subsequent passages.

The necessity for gradually increasing the age of the rabbits being used for successful infection of older animals is apparent.

Experiments in this laboratory [6] had shown that cortisone acetate decreased the resistance of adult mice to foot-and-mouth disease virus. Accordingly, adult rabbits weighing 2.5 kg. were inoculated subcutaneously with 100 mg. of cortisone and, five hours later, with virus of the twentieth passage of the O type strain. These rabbits, as well as control rabbits of the same age and weight which were not given cortisone, remained normal.

Incubation Period and Clinical Signs of Disease.—During the earlier passages in extremely young rabbits, the incubation period varied from 40 to 72 hours but, in subsequent passages, it gradually decreased to less than 24 hours. In rabbits 14 days old and older, the incubation period never was less than 24 to 72 hours.

The first sign of the disease is a reduction in activity, with evidence of increasing weakness in the limbs. In the case of extremely young rabbits, the sick animal usually is found separated from the rest of the litter. Increased weakness, paralysis, and the cessation of sucking lead to death in one to four days in the earlier passages but, in the case of 1- to 2-day-old rabbits from the tenth passage on, death occurs within 24 hours. Older rabbits die within two to five days.

In 1- to 2-day-old rabbits, the mortality was 100 per cent. Occasionally, older rabbits survived the inoculation of virulent material.

Pathogenicity of Virus Passaged in Rabbits for Cattle.—Undiluted or diluted suspensions of muscle from passages 5, 20, 40, 96, and 111 of the O type strain were inoculated intradermally into the tongues of cattle. Each suspension was titrated in unweaned mice.

The age of the rabbits used, the amount of virus, the number of sites inoculated, and the number of cattle and their reactions are given (table 3). Virus suspensions prepared at different dates from muscles of rabbits in the same group were used in the first two experiments with the 111th passage material.

In the last two experiments, material from a second 111th passage was used. No loss of pathogenicity occurred up to the fortieth passage; however, 11 of the 19 cattle inoculated with ninety-sixth or 111th passage material had no reactions, and only 3 of the remaining 8 had complete generalized infections.

Similar experiments were done, using the twentieth and fortieth passages of the A and C type strains (table 3). Although the number of cattle used was small, it seems certain that little loss of pathogenicity for cattle has occurred, at least for the A type strain.

Infective tongue epithelium was collected from all cattle that developed local lesions

XUM

TABLE 3—Reactions of Cattle to Intradermal Tongue Inoculation of Rabbit-Passaged Virus

Virus type	Passage No.	Age of rabbits (days)	No. sites and dose of virus inoculated (i.d.$_{50}$)	No. cattle inoculated and their reactions
O	5	2	$4 \times 10^{4.8}$	2 (T, 4F)
	20	2	$4 \times 10^{5.9}$	2 (T, 4F)
	40	2	$4 \times 10^{6.3}$	2 (T, 4F)
	96	24	$20 \times 10^{5.1}$	1 (T), 1 (T, 2F)
	111	40	$10 \times 10^{6.7}$	3 (Neg.), 1 (T, 4F)
	111	40	$10 \times 10^{6.3}$	3 (T, 2F), 2 (T, 4F)
	111	40	$10 \times 10^{4.6}$	4 (Neg.)
	111	40	$10 \times 10^{4.8}$	4 (Neg.)
A	20	2	$6 \times 10^{2.8}$	1 (T, 4F)
	20	2	$6 \times 10^{3.8}$	1 (T, 4F)
	40	15	$6 \times 10^{4.8}$	3 (T, 4F)
C	20	2	$10 \times 10^{3.9}$	2 (T, 4F)
	40	15	$4 \times 10^{5.7}$	1 (T)
	40	15	$4 \times 10^{4.7}$	1 (T)

T = tongue; F = foot; 2F = two feet, etc.

for determination of the virus type by the complement-fixation test. In each case, the results showed that the material examined was of the same immunological type as that used originally.

With the exception of the 4 cattle used in the first two experiments with the O type strain, the antibody content of the serum of the cattle was determined before the inoculation of rabbit virus. In no animal was there a significant level of antibody. Antibody levels after inoculation and after the challenge of immunity are included in the following paper,[8] describing the immunogenicity of the virus passaged in rabbits.

Discussion

Although other authors [11, 16, 18, 23] have described the susceptibility of the young rabbit to inoculation with foot-and-mouth disease virus, this paper reports the successful direct passage of the virus through a longer series and the successful infection of older rabbits. Serial passage in older rabbits with the type O strain has resulted in a marked reduction in the pathogenicity of the virus for cattle. The initial susceptibility of the cattle used was proved by the absence of a significant level of antibody, as determined by serum-protection tests in unweaned mice.

No attempt has been made to substitute the unweaned rabbit for the unweaned mouse as an indicator of the presence of foot-and-mouth disease virus. This would present obvious disadvantages. However, if the object is to use the animal to obtain infective material, a young rabbit yields more than a young mouse. The possibility of using infective rabbit tissue as a source of virus for vaccine production was the initial purpose of the investigation. The discovery that high serial passage is accompanied by a reduction of pathogenicity for cattle suggested the even more valuable application of the modified strain as a live-virus vaccine.

Summary

One strain each of the Vallée O, Vallée A, and Waldmann C types of foot-and-mouth disease virus was adapted to the young rabbit by serial passage. The 137th, fortieth, and fortieth passages, respectively, were reached.

Initially, 1- to 2-day-old rabbits were used but, by gradually increasing the age of the rabbits during the course of passaging the virus, it was possible to infect 51-day-old rabbits with the O type strain from the 112th passage on.

With the O type strain, high serial passage was accompanied by a marked reduction in the pathogenicity for cattle.

References

[1] Bedson, S. P., Maitland, H. B., and Burbury, Y. M.: Second Report of the Foot-and-Mouth Disease Committee. H.M. Stationery Off. (London), (1927): 104–105.

[2] Burbury, Y. M.: Third Report of the Foot-and-Mouth Disease Committee. H.M. Stationery Off. (London), (1928): 70–77.

[3] Camargo, F. N., Eichhorn, E. A., Levine, J. M., and Tellez-Giron, A.: A Complement Fixation Technique for Foot-and-Mouth Disease and Vesicular Stomatitis. Proc. Book, AVMA (1950): 207–211.

[4] Cunha, R. G.: Organization of the Pan-American Center Against Foot-and-Mouth Disease and Initial Research. FAO Development Paper 33, Rome, 1953 (Summary in Rep. 2nd Inter-Am. Meet. Livestock Production, Bauru, 1952).

[5] Cunha, R. G., Baptista, J. A., Jr., Serrão, U. M., and Torturella, I: El uso de los ratones lactantes en la avaluación de los anticuerpos contra el virus de la fiebre aftosa y su significación inmunológica. Gac. vet., *19*, (1957): 243–267.

[6] Cunha, R. G., and Eichhorn, E. A.: Influence of Cortisone on Susceptibility of Adult Mice to Foot-and-

Mouth Disease Virus. Am. J. Vet. Res., *15*, (Jan., 1954): 149–151.

[7] Cunha, R. G., Martins, I. A., Carvalho, J. M., and Passos, W. S.: Estudo comparativo de metodos de preparo de antigeno para tipificação de virus aftoso pela prova de fixacão de complemento. Arch. Inst. biol. anim., *1*, (1950): 31–48.

[8] Cunha, R. G., Torturella, I., and Serrão, U. M.: Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus. II. Immunogenicity. Am. J. Vet. Res., *20*, (Jan., 1959): 138–144.

[9] Dacorso Filho, Paulo, and Cunha, R. G.: Lesões observadas em coelhos recem nascidos inoculados com amostras de três tipos de virus de febre aftosa. Rev. bras. Biol., *14*, (1954): 343–353.

[10] Eichhorn, E. A.: Laboratory Diagnosis of Foot-and-Mouth Disease. Proc. 15th Internat. Vet. Cong. (Stockholm), *1*, (1953): 187–191.

[11] Garcia Mata, E., Federer, K. E., Pizza, L., and Aramburu, H. G.: Acción patogena del virus aftoso en neonatos de diferentes especies. Gac. vet., *17*, (1955): 57–64.

[12] Garcia Mata, E., Pizza, L., and Aramburu, H.: El cultivo del virus aftoso en el ratón y en la rata blanca. Gac. vet., *13*, (1951): 74.

[13] Gibbs, Y.: Fourth Report of the Foot-and-Mouth Disease Committee. H.M. Stationery Off. (London), (1931): 164.

[14] Gillespie, H.: The Propagation and Effects of Type "A" Foot-and-Mouth Disease Virus in the Day Old Chick. Cornell Vet., *44*, (1954): 425–432.

[15] Gioliti, G.: Ricerche sull'afta maliga. I. La riproduzione sperimentale del 'cor tigratum' nel cane. Arch. vet. ital., *1*, (1950): 253–281.

[16] Gribanov, V.: Résultats de l'épreuve du vaccin antiaphteux VIEV préparé avec un virus adapté au lapin. Off. Internat. des Épizoot., *43*, (1955): 632–635.

[17] Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. Agric. Res. Council Rep. Series 9, H.M. Stationery Off. (London), 1949.

[18] Nagel, H. C.: El comportamiento del virus aftoso en animales lactantes de diferentes especies. Gac. vet., *14*, (1952): 52–59.

[19] Pan American Foot-and-Mouth Disease Center: Annual Reports for the Years 1952, 1953, 1954, 1955, and 1956. Pan American Sanitary Bureau, Washington, D.C.

[20] Reed, L. T., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[21] Schmidt Funes, E.: Receptividad del hamster al virus de la fiebre aftosa. Gac. vet., *15*, (1953): 157–160.

[22] Skinner, H. H.: Propagation of Strains of Foot-and-Mouth Disease Virus in Unweaned White Mice. Proc. Roy. Soc. Med., *44*, (1951): 1041–1044.

[23] Verge, J., Paraf, A., Dhennin, L., Dhennin, L., and Asso, J.: Culture du virus aphteux sur le lapin nouveau-né. Modification des pouvoirs pathogènes et antigénes. Bull. Acad. vét., *30*, (1957): 291–298.

[24] Waldmann, O., Nagel, H. C., and Zimmermann, Th.: L'infection aphteuse artificielle chez les veaux nouveaux-nés à jeunes. Off. Internat. des Épizoot., *43*, (1955): 708–722.

[25] Waldmann, O., and Trautwein, K.: Maul- und Klauenseuche. In "Handbuch der pathogenen Mikroorganismen." (by Kolle, W., Krauss, R., and Uhlenhuth, P.) 3rd ed., vol. 9. Gustav Fischer und Urban und Schwarzenberg, Jena, Germany, 1929.

XUM

Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus
II. Immunogenicity

RAYMUNDO G. CUNHA, D.V.M.; IVO TORTURELLA, D.V.M.;
UBIRATAN M. SERRÃO, D.V.M.

Rio de Janeiro, Brazil

THE PRECEDING PAPER [2] reported the adaptation of strains of foot-and-mouth disease virus to the rabbit, the distribution of the virus in the rabbit, and the diminution with serial passage of the pathogenicity of the virus for cattle.

This paper describes the results of attempts to immunize cattle, using the rabbit-adapted virus for preparation of inactivated-virus vaccine and also as a modified live-virus vaccine.

MATERIALS AND METHODS

The description of materials and methods supplements that in the preceding paper.[2]

Strains of Foot-and-Mouth Disease Virus—Rabbit-Adapted Virus.—The Nilopolis strain, Vallée O type, was used throughout these experiments.

Bovine Virus.—The virus used for challenge of the vaccinated cattle was the Barra Mansa strain, Vallée O type, isolated in 1955 from an outbreak of the disease in the state of Rio de Janeiro, Brazil. It has since been maintained by serial passage in cattle.

Preparation of Inactivated-Virus Vaccine.—The standard type of Schmidt-Waldmann formolized aluminium hydroxide vaccine was prepared, using a suspension of ground rabbit muscle clarified by centrifugation. The virus suspension was titrated in unweaned mice. The pH of the vaccine was corrected and maintained at 7.6 to 7.8 by the use of glycocol buffer. The final concentration of aluminium hydroxide gel* was 50 per cent; batches of 3 to 4 liters were prepared.

Each batch of vaccine was tested for non-infectivity by tongue and subcutaneous inoculation of 2 cattle and by the intraperitoneal inoculation of 8 unweaned mice.

TABLE 1—Results of Vaccination Experiments in Cattle Using Inactivated-Virus Vaccine Prepared from Virus of the Fifth Passage in Rabbits

Animal (No.)	Dose of vaccine (ml.)	Original virus content of vaccine dose (i.d.$_{50}$)	Method of challenge*	Lesions
A12	5	10^8	Contact	Neg.
A3	5	10^8	Contact	T, M, 4F
A6	5	10^8	Inoculation	T
A9	5	10^8	Inoculation	T, 1F
A60	10	$10^{8.3}$	Contact	Neg.
A30	10	$10^{8.3}$	Inoculation	T, 4F
A90	10	$10^{8.3}$	Inoculation	T, 4F
A40	10	$10^{8.3}$	Inoculation	T, M, 4F
A80, control	....	....	Contact	T
A82, control	....	....	Contact	T, 3F
A85, control	....	....	Inoculation	T, 2F
A88, control	....	....	Inoculation	T, 4F

* Challenge was 16 days after vaccination. T = tongue; M = elsewhere in mouth; F = foot.

Modified Live-Virus Vaccine.—Muscle from infected rabbits was ground, suspended in phosphate buffer solution, and clarified by centrifugation. In most instances, the muscle tissue had been harvested earlier, finely chopped and mixed, and stored at −20 C. The virus suspension was titrated in unweaned mice.

Vaccination of Cattle.—Inactivated vaccine was inoculated subcutaneously in the lower part of the neck. Live-virus vaccine was inoculated intradermally on the tongue or intracutaneously on the cheek.

The cattle used in the controlled experiments were from 14 to 18 months old. In field trials, cattles of various ages were used. All animals were crossbred, with Zebu characteristics predominating.

From the Pan American Foot-and-Mouth Disease Center, Pan American Sanitary Bureau, Rio de Janeiro, D.F., Brazil.

The authors thank Dr. W. M. Henderson, director of the Center, for his criticism and review of the text; Dr. J. L. Saile, for performing most of the complement-fixation tests; and Messrs. Antonio Salles and José Ribamar, for assisting with the inoculation and observation of the experimental animals.

* Provided by the Instituto de Biologia Animal of the Brazilian Ministry of Agriculture.

TABLE 2—Results of Vaccination Experiments in Cattle Using Inactivated-Virus Vaccine Prepared from Virus of a Second Fifth Passage in Rabbits

Animal (No.)	Dose of vaccine (ml.)	Original virus content of vaccine dose (i.d.$_{50}$)	SNI 0 days	SNI 20 days	Method of challenge*	Lesions	SNI 41 days
21	10	$10^{6.7}$	0.8	2.0	Contact	T, 3F	>5.7
22	10	$10^{6.7}$	1.7	1.7	Contact	T, M, 4F	>5.7
23	10	$10^{6.7}$	1.7	3.0	Inoculation	T, 4F	>5.7
24	10	$10^{6.7}$	1.3	2.7	Inoculation	T, 4F	>5.7
25	20	$10^{7.0}$	1.8	2.2	Contact	M, 3F	>5.7
26	20	$10^{7.0}$	1.3	5.0	Contact	Neg.	>5.7
27	20	$10^{7.0}$	0.8	2.7	Inoculation	T	>5.7
28	20	$10^{7.0}$	1.7	2.2	Inoculation	T, M, 4F	>5.7
29, control	....	....	....	1.7	Inoculation	T, 4F	>5.7
210, control	....	....	....	1.7	Inoculation	T, 4F	>5.7
211, control	....	....	....	1.2	Contact	T, 4F	>5.7
212, control	....	....	....	1.8	Contact	T, 4F	>5.7

* Challenge was 20 days after vaccination.
SNI = serum-neutralization indexes; T = tongue; M = elsewhere in mouth; F = foot.

Challenge of Vaccinated Cattle—Challenge by Inoculation.—Vaccinated and control cattle were inoculated intradermally on the tongues with a suspension of the bovine virus strain. One site of inoculation was used. The criterion for protection was the absence of secondary lesions. The challenge virus was titrated in cattle by Henderson's method,[4] using chloral hydrate anesthesia.

Challenge by Contact.—Vaccinated and control cattle were left in contact with the inoculated cattle. The criterion for protection was the absence of all lesions.

Examination of Cattle Serums for the Presence of Antibody.—The antibody content of serum samples was determined by either the serum-neutralization or the serum-protection test in unweaned mice. Details of the techniques used and an appraisal of the comparative values of the two tests have been published.[1]

RESULTS

Inactivated-Virus Vaccine.—One batch of vaccine was prepared from muscle of each of the fifth, a second fifth, and the thirty-sixth passages in rabbits.

In the tests of noninfectivity, 2 ml. of vaccine was inoculated intradermally into the tongue of each of 2 cattle. The subcutaneous dose was 15 ml., 20 ml., and 100 ml., respectively, of each batch. The mice were inoculated with 0.05 ml. intraperitoneally. In no case was a positive reaction obtained.

In testing the second and third batches of vaccine, blood samples were taken from all cattle, and the antibody content of each sample was determined.

Details about the vaccines, antibody levels, and results of the challenge of the vaccinated and control cattle are given (tables 1–3).

In the first two experiments, although

TABLE 3—Results of Vaccination Experiments in Cattle Using Inactivated-Virus Vaccine Prepared from Virus of the Thirty-sixth Passage in Rabbits

Animal (No.)	Dose of vaccine (ml.)	Original virus content of vaccine dose (i.d.$_{50}$)	SNI 0 days	SNI 20 days	Method of challenge*	Lesions	SNI 41 days
31	25	$10^{8.9}$	1.1	3.0	Inoculation	T, M, 4F	2.8
32	25	$10^{8.8}$	3.7	>4.5	Inoculation	Neg.	>4.7
33	25	$10^{8.8}$	1.5	2.5	Contact	T, 4F	>4.7
34	25	$10^{8.8}$	1.2	2.8	Contact	T, 4F	3.0
35	50	$10^{9.1}$	1.7	2.0	Inoculation	T, 4F	2.2
36	50	$10^{9.1}$	0.8	0.8	Inoculation	T, 4F	2.0
37	50	$10^{9.1}$	4.5	>4.5	Contact	Neg.	>4.7
38	50	$10^{9.1}$	0.8	1.5	Contact	T, M, 4F	3.0
39	100	$10^{9.4}$	2.5	>4.5	Inoculation	Neg.	>4.7
310	100	$10^{9.4}$	3.5	>4.5	Inoculation	Neg.	>4.7
311	100	$10^{9.4}$	2.5	>4.5	Contact	Neg.	>4.7
312	100	$10^{9.4}$	1.5	1.5	Contact	T, M, 4F	3.2
313, control	....	....	....	1.2	Inoculation	T, 4F	>4.7
314, control	....	....	....	1.7	Inoculation	T, 1F	3.7
315, control	....	....	....	0.8	Contact	T, 4F	2.0
316, control	....	....	....	4.6	Contact	Neg.	>4.7
317, control	....	....	....	1.2	Inoculation	T, 4F	>4.7
318, control	....	....	....	0.8	Inoculation	T, 4F	>4.7

* Challenge was 20 days after vaccination.
SNI = serum-neutralization indexes; T = tongue; M = elsewhere in mouth; F = foot.

XUM

TABLE 4—Responses of Cattle to Intradermal Tongue Inoculation and the Results of Challenge by Tongue Inoculation with Bovine Virus (10^3 i.d.$_{50}$) at 22 Days *

Animal (No.)	No. sites and dose of rabbit virus inoculated (i.d.$_{50}$)	Reaction	SPI 0 days	SPI 8 days	SPI 15 days	SPI 22 days	Result of challenge	SPI 36 days
138	$10 \times 10^{6.7}$	Neg.	1.0	>5.5	>5.5	>5.5	Neg.	>5.8
139	$10 \times 10^{6.7}$	Neg.	0	2.2	3.3	4.1	T	>5.8
145	$10 \times 10^{6.7}$	Neg.	0.5	2.0	>5.5	5.3	T	>5.8
149	$10 \times 10^{6.7}$	T, 4F	0	>5.5	>5.5	>5.5	Neg.	>5.8
119, control	.....	....	....	....	....	1.1	T, 4F	>5.8
150, control	.....	....	....	....	....	1.2	T, 4F	>5.8

* Experiment 1, using modified live virus of the 111th rabbit passage.
SPI = serum-protection indexes; T = tongue; F = foot.

the number of cattle used was small, there was an indication that the rabbit virus was of little value for the preparation of the standard type of inactivated-virus vaccine.

In the third experiment, all cattle that resisted challenge initially had significantly high levels of antibody, and these results must be discarded. There was, therefore, no evidence of protection, even with the massive quantities of virus used in the preparation of the third batch of vaccine.

Modified Live-Virus Vaccine—Controlled Experiments.—Four experiments were performed with rabbit-adapted virus of the 111th passage (tables 4–7). Intradermal tongue and intracutaneous inoculations were used. In three of the experiments, a group of cattle was left in contact with those that had been vaccinated. Blood samples were collected during the course of these experiments. In no animal was there an initial significant level of antibody, proving that they were susceptible.

The reactions to rabbit-adapted virus of cattle inoculated intradermally on the tongue also were given in the preceding paper,[2] in which the pathogenicity of the virus is described. A study of the results obtained in determining the antibody content of the serums of these cattle during the experiments shows that, in general, the inoculation of rabbit virus was followed either by a marked increase in antibody, similar to that in a clinically infected animal, or by a relatively small increase. This suggests that, in the first case, a subclinical infection was established by the modified strain, with multiplication of virus to produce large quantities of antigen. In the other case, the antibody simulation would be that resulting only from the initial inoculation of antigen. This lower level was, however, sufficient to protect the cattle at the time of challenge but, without further stimulus, a long duration of immunity probably could not be expected.

TABLE 5—Responses of Cattle to Tongue and Intracutaneous Inoculation and Results of Challenge by Tongue Inoculation with Bovine Virus (10^3 i.d.$_{50}$) at 21 Days *

Animal (No.)	No. sites and dose of rabbit virus inoculated (i.d.$_{50}$)	Route of inoculation	Reaction	SPI (days) 0	7	14	21	Result of challenge	SPI 36 days
162	$10 \times 10^{6.3}$	T	T, 2F	0.5	>6.0	>6.0	>6.0	Neg.	>5.7
163	$10 \times 10^{6.3}$	T	T, 2F	0.7	>6.0	>6.0	>6.0	Neg.	>5.7
167	$10 \times 10^{6.3}$	T	T, 2F	0.0	>6.0	>6.0	>6.0	Neg.	>5.7
155	$10 \times 10^{6.3}$	T	T, 4F	0.5	>6.0	>6.0	>6.0	Neg.	>5.7
168	$10 \times 10^{6.3}$	T	T, 4F	0.5	0.7	1.3	1.3	T, 1F	>5.7
154	.... $10^{7.6}$	i.c.	Neg.	0.8	>6.0	>6.0	>6.0	Neg.	>5.7
164	.... $10^{7.6}$	i.c.	Neg.	1.0	>6.0	>6.0	>6.0	Neg.	>5.7
166	.... $10^{7.6}$	i.c.	Neg.	1.0	>6.0	>6.0	>6.0	Neg.	>5.7
157	.... $10^{7.6}$	i.c.	Neg.	0.5	0.5	0.5	1.4	T	>5.7
159	.... $10^{7.6}$	i.c.	Neg.	0.5	>6.0	>6.0	>6.0	T	>5.7
169	.....	Contact	Neg.	0.5	0.3	1.0	0.8	T, 2F	>5.7
160	.....	Contact	Neg.	0.7	1.0	0.2	0.5	T, 4F	>5.7

* Experiment 2, using modified live virus of the 111th rabbit passage.
SPI = serum-protection indexes; T = tongue; F = foot; i.c. = intracutaneous.

The reactions to rabbit-adapted virus of cattle inoculated intradermally on the tongue also were given in the preceding paper,[2] in which the pathogenicity of the

Seven of 17 cattle (41%) inoculated in the tongue with rabbit-adapted virus reacted with clinical infections. Of 21 inoculated intracutaneously, 2 (10%) reacted clinically. Of the 29 nonreactors from both groups, 23 (79%) were protected on challenge with bovine virus. Of 10 cattle left in contact with those vaccinated with rabbit-adapted virus, only 2 showed signifi-

TABLE 6—Responses of Cattle to Tongue and Intracutaneous Inoculation and the Results of Challenge by Tongue Inoculation of Bovine Virus (10^3 i.d.$_{50}$) at 21 Days *

Animal (No.)	No. sites and dose of rabbit virus inoculated (i.d.$_{50}$)	Route of inoculation	Reaction	SPI (days) 0	7	14	21	Result of challenge	SPI 36 days
202	$10 \times 10^{4.6}$	T	Neg.	0.3	0.5	1.2	2.2	Neg.	>5.5
204	$10 \times 10^{4.6}$	T	Neg.	1.0	>5.2	>5.2	>6.0	Neg.	>6.0
206	$10 \times 10^{4.6}$	T	Neg.	0.2	3.0	4.8	2.2	T	>5.5
214	$10 \times 10^{4.6}$	T	Neg.	0.5	0.2	1.0	1.7	T, 4F	>6.0
201	.... $10^{6.9}$	i.c.	Neg.	1.0	>5.2	>5.2	5.0	T	>6.0
208	.... $10^{6.9}$	i.c.	Neg.	0.5	3.0	2.5	2.5	Neg.	3.0
212	.... $10^{6.9}$	i.c.	Neg.	0.7	>5.2	>4.8	>6.0	Neg.	>6.0
215	.... $10^{6.9}$	i.c.	T, 3F	0.3	>5.2	>6.1	>6.0	Neg.	>6.0
205	.... $10^{6.9}$	i.c.	Neg.	0.3	1.4	2.0	3.0	T	>6.0
207	.... $10^{6.9}$	i.c.	Neg.	0.5	0.2	1.0	0.9	T	>6.0
209	.... $10^{6.9}$	i.c.	Neg.	0.0	0.0	0.0	0.8	T, 4F	>6.0
210	.... $10^{6.9}$	i.c.	Neg.	0.5	0.4	1.7	0.5	T, 4F	>4.5
203†	.....	Contact	Neg.	0.3	0.5	3.2	4.1	T	>6.0
211†	.....	Contact	Neg.	0.4	0.0	0.7	0.2	T, 4F	>5.5
213†	.....	Contact	Neg.	0.5	0.0	0.3	0.5	T, 2F	>5.5
216†	.....	Contact	Neg.	0.5	0.0	0.3	1.0	T, 4F	>6.1
220, control**	.....	....	....	....	....	....	0.2	T, 2F	>5.3
217, control**	.....	....	....	....	....	....	0.7	T, 4F	>5.3
218, control**	.....	....	....	....	....	....	0.0	T, 4F	>5.3
219, control**	.....	....	....	....	....	....	0.4	T, 4F	>5.3

* Experiment 3, using modified live virus of the 111th rabbit passage; † uninoculated, but kept in contact with inoculated animals: ** uninoculated and had no contact with inoculated animals.
SPI = serum-protection indexes; T = tongue; F = foot; i.c. = intracutaneous.

cant resistance to challenge and in only 1 was there any rise in antibody during this period of contact (serum-protection indexes of 3.2 at 14 days and 4.1 at 21 days).

Field Trials.—Following the promising results that were obtained in the controlled experiments with rabbit-adapted virus of the 111th passage, virus of the same passage was used in preliminary trials in the field. Five farms in the neighborhood of Rio de Janeiro were selected and a total of 142 animals was vaccinated. Because of the incidence of foot-and-mouth disease in this area, the cattle also were vaccinated with Schmidt-Waldmann formolized aluminium hydroxide vaccine* against virus types A and C after a period of not less than one month after the rabbit virus inoculation.

The modified live-virus vaccination consisted of the intracutaneous inoculation of 2 ml. of a 1:9 virus suspension. According to the results of titrations in suckling mice, this dose of virus was $10^{6.4}$, $10^{6.1}$, $10^{7.1}$, $10^{6.1}$, and $10^{6.5}$ i.d.$_{50}$ for animals on farms 1 to 5, respectively. Two cattle on farm 4 developed lesions of foot-and-mouth disease during the second week. The initial

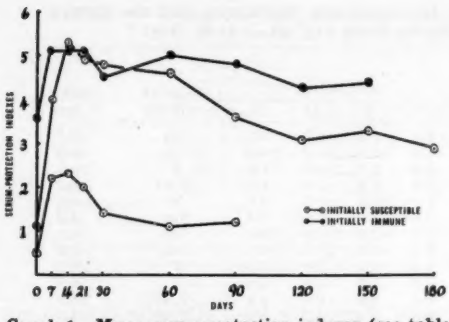
* Obtained from the Instituto de Biologia Animal of the Brazilian Ministry of Agriculture.

TABLE 7—Responses of Cattle to Tongue and Intracutaneous Inoculation and the Results of Challenge by Tongue Inoculation of Bovine Virus ($10^{2.5}$ i.d.$_{50}$) at 21 Days *

Animal (No.)	No. sites and dose of rabbit virus inoculated (i.d.$_{50}$)	Route of inoculation	Reaction	SPI (days) 0	7	14	21	Result of challenge	SPI 36 days
230	$10 \times 10^{4.8}$	T	T erosion	0.9	3.8	....	>5.5	Neg.	5.1
226	$10 \times 10^{4.8}$	T	Neg.	0.0	0.8	5.5	4.3	Neg.	>5.6
234	$10 \times 10^{4.8}$	T	Neg.	0.5	>5.3	>5.3	>5.5	Neg.	>5.6
245	$10 \times 10^{4.8}$	T	Neg.	0.5	4.3	>5.3	>5.5	Neg.	>5.6
252	.... $10^{7.1}$	i.c.	T, 4F	0.5	>6.0	>6.0	>6.0	Neg.	>5.6
250	.... $10^{7.1}$	i.c.	Neg.	1.0	>6.0	>6.0	4.1	Neg.	>3.8
237	.... $10^{7.1}$	i.c.	Neg.	0.0	0.2	1.0	1.2	T	>5.6
238	.... $10^{7.1}$	i.c.	Neg.	0.5	0.5	1.1	1.9	T	>5.6
244	.... $10^{7.1}$	i.c.	Neg.	0.0	0.8	1.0	1.7	T	>5.6
241	.... $10^{7.1}$	i.c.	Neg.	0.0	0.0	0.8	0.0	T, 2F	>5.6
242	.... $10^{7.1}$	i.c.	Neg.	0.0	0.0	1.0	1.0	T, 4F	>5.6
243	.... $10^{7.1}$	i.c.	Neg.	0.3	1.0	1.1	0.5	T, 4F	>5.6
249†	.....	Contact	Neg.	1.0	0.8	0.3	....	Neg.	>5.6
240†	.....	Contact	Neg.	0.3	0.3	0.0	0.5	T, 2F	>5.6
239†	.....	Contact	Neg.	0.2	0.7	1.3	0.0	T, 4F	>5.6
248†	.....	Contact	Neg.	0.0	0.0	0.0	0.5	T, 4F	>5.6

* Experiment 4, using modified live virus of the 111th rabbit passage; † uninoculated, but kept in contact with inoculated animals.
SPI = serum-protection indexes; T = tongue; F = foot; i.c. = intracutaneous.

XUM



Graph 1—Mean serum-protection indexes (see table 8) of groups of cattle on farms 1 and 2, during the field trial of the modified live-virus vaccine. See table 9 for the number of cattle involved.

degree of susceptibility of the cattle and the subsequent development of immunity was determined by the collection and testing of serum samples for the presence of O type antibody. This was possible for all but 14 of the 142 cattle vaccinated.

Five months after vaccination, the disease was observed in 2 cattle on farm 3. The virus strain isolated, however, was of the Vallée A type, eliminating the rabbit-adapted virus vaccine. The disease was not known to occur in other animals during six months of observation.

On the basis of the initial serum-protection indexes, the cattle were assigned to two groups. Those with an index of 2.0 or greater, which should be resistant on challenge, were classified as "initially immune"; the remainder, most of which had an index of about 1.0, were classified as "initially susceptible."

Tests were made of the serums of all the animals on farms 1 and 2 at 0, 7, 14, 21, and 30 days and at two, three, four, five, and six months (table 8). On other farms, these tests were made only at monthly intervals.

The initially susceptible cattle can be assigned to groups of those that did and did not show an appreciable antibody response at, for instance, two months. Table 8 has been arranged to show this grouping, as well as a grouping of those initially immune. Vaccination was followed by a rise in antibody titer for all animals in this last group on all farms. A few susceptible animals showed transient responses that disappeared during the first month; the significance of this has not been discovered.

The average serum protection index of each group is shown graphically (graph 1). Unfortunately, the collection of blood samples from 3 cattle showing no immune responses was not continued after 60 days. The mean indexes for groups 2 have not, therefore, been calculated beyond this point.

A similar grouping was obtained from the results on each of the other farms, with

TABLE 8—Field Trials of Modified Live Virus of the 111th Rabbit Passage—Serum-Protection Indexes of the Vaccinated Cattle on Farms 1 and 2

Group	Animal (No.)	Serum protection indexes (days)									
		0	7	14	21	30	60	90	120	150	180
I	3	1.5	>4.6	>5.8	4.1	>5.5	4.4	3.2	2.5	3.0	2.3
(initially susceptible with response)	4	0.2	>4.6	>5.5	>5.6	>5.6	>5.5	>5.5	5.0	3.2	2.7
	7	1.5	>4.6	>5.8	>5.6	>5.0	5.5	2.5	2.0	4.5	>5.5
	10	2.2	4.1	4.5	4.1	3.8	4.0	1.8	2.5	2.5	2.5
	22	0.5	>4.7	>4.7	>5.8	>5.8	>6.0	>5.5	4.5	2.5	3.3
	23	1.5	>4.7	>4.7	3.1	2.2	3.5	3.5	3.5	4.9	2.7
	25	0.2	0.5	>5.8	>5.8	>6.0	3.5	3.5	1.8	2.2	1.1
Mean		1.1	4.0	5.3	4.9	4.8	4.6	3.6	3.1	3.3	2.9
II	1	0.2	1.0	1.6	1.0	1.7	1.2	0.9	1.5	1.7	1.5
(initially susceptible without response)	2	1.0	>4.6	5.0	3.2	2.5	2.2	1.9	2.0	2.2	1.8
	5	1.0	3.0	4.0	1.0	1.8	1.0	0.9	1.7	2.2	2.0
	8	0.5	3.1	>5.8	5.6	2.8	2.0	2.0	1.0	2.8	3.0
	9	2.0	0.7	1.0	0.9	0.5	0.0	0.0	0.8	2.0	1.3
	13	0.2	>6.0	3.0	2.6	3.0	2.3	1.3	0.9	2.5	....
	18	0.0	0.5	0.5	0.6	0.0	0.0	....	....	....	....
	19	0.0	0.0	0.0	0.0	0.0	0.0	....	....	....	....
	20	0.0	0.5	0.9	0.8	0.0	0.0	....	....	....	....
	21	0.8	>4.7	3.5	5.8	2.6	2.3	1.8	0.5	1.9	1.7
	24	0.0	0.5	0.0	0.6	0.3	1.5	0.8	0.5	0.3	0.1
Mean		0.5	2.2	2.3	2.0	1.4	1.1	....	....	....	....
III	6	3.3	>4.6	>5.8	>5.6	>5.0	>5.6	4.0	2.9	2.8	3.0
(initially immune)	11	3.8	>5.5	4.3	>4.6	>4.0	4.3	>5.6	>5.6	>6.0	....
Mean		3.6	5.1	5.1	5.1	4.5	5.0	4.8	4.3	4.4	....

some variation between the proportion of animals in each group (table 9).

The average protection indexes, at monthly intervals, of all groups on all farms are shown (graph 2).

The antibody titer in the groups of cattle which showed responses remained high enough throughout the six-month observation period to indicate a degree of immunity that would give complete protection against the disease.

Discussion

Although these experiments on the preparation of aluminium hydroxide vaccines, using tissue from unweaned rabbits, are not extensive, the results do not indicate that rabbit-passaged virus would be likely to be of value in this connection. This is contrary to the results of Gribanov,[3] who reported satisfactory results using rabbit tissue.

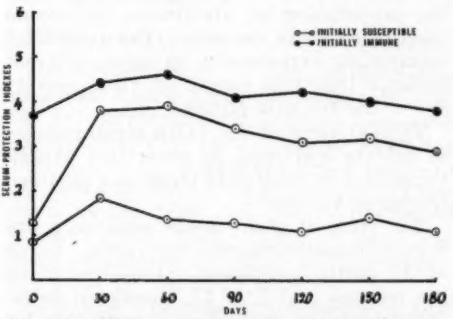
Conflicting reports also exist on the use of virus passaged in unweaned mice. For example, Skinner *et al.*[7] found that a mouse virus vaccine apparently gave better protection than a bovine virus vaccine, judging by the original virus content of the vaccines, whereas Waldmann and Nagel [8] concluded that mouse virus vaccines were of no value for the immunization of cattle.

The results obtained with rabbit-passaged virus as a modified live-virus vaccine are more promising. The degree of immunity produced would appear satisfactory, as indicated by the persistence of an antibody titer adequate for protection for at least six months. In the field trials, however, the proportion of cattle in which an immune response occurred was small—no more than one third of those susceptible initially. This proportion may be influenced by such factors as the virus dose, the number of doses, the route of inoculation, and the state of the animal—all of which are subjects for further investigation.

A low degree of pathogenicity still exists with the passages that have been made, but this does not necessarily render the use of such a strain impractical in countries where the disease is enzootic.

In presenting the results of the controlled experiments with live virus, we suggested that the substantial rise in antibody titer that occurred in some animals was the result of the establishment of a subclinical infection. Pay [6] found that virus without pathogenicity for cattle, following serial passage in mice or in embryonating eggs, still was capable of multiplying in monolayer cultures of bovine tongue epithelial cells, but without the production of any cytopathogenic effect. He also stated that certain attenuated strains may fail to multiply in such cultures.

Our hypothesis is, therefore, that the production of immunity to foot-and-mouth



Graph 2—Mean serum-protection indexes of groups of cattle from all farms during the field trial of the modified live-virus vaccine. See table 9 for the number of cattle involved.

disease, following the use of a modified live-virus vaccine, probably is dependent on the establishment of infection with virus multiplication—multiplication that is, however, not necessarily cytopathogenic.

The possibility of spread of a modified live virus must be considered. For example, Huck and Hudson [5] found that a lapinized strain of hog cholera virus was unsuitable for use in the field, since the virus could spread from vaccinated to unvaccinated swine, with the production of lesions of the disease. In our experiments, there was little tendency for the virus to spread, even when the clinical disease occurred in some cattle inoculated with the rabbit virus.

TABLE 9—Field Trials of Modified Live Virus of the 111th Rabbit Passage—Summary of Results for 128 of 142 Cattle Vaccinated on Five Farms

Item	Farms				Totals
	1 and 2	3	4	5	
No. cattle	20	32	45	31	128
No. initially susceptible	18	12	37	18	85
No. responding to vaccine	7	6	8	8	29
No. initially immune	2	20	8	13	43

Two thirds of the cattle were initially susceptible and one third of these became immunized following vaccination.

Until the effect of this modified strain of virus on young calves or on pregnant cows is investigated, our field work is being restricted, in case the fatal muscle necrosis syndrome of the unweaned mouse or rabbit should occur following infection of the bovine fetus or calf.

SUMMARY

Foot-and-mouth disease virus adapted to the rabbit by serial passage was used for the preparation of aluminium hydroxide inactivated-virus vaccines. The results of vaccination experiments in cattle did not indicate that this source of virus was of any value for this purpose.

Type O virus of the 111th serial passage in rabbits was used in controlled experiments and in small field trials as a modified live-virus vaccine.

The virus of this passage retained a low degree of pathogenicity for cattle. Seven of 17 cattle inoculated intradermally on the tongues and 2 of 21 inoculated intracutaneously in controlled experiments became clinically infected, as did 2 of 142 cattle inoculated intracutaneously in field trials.

The disease did not appear in cattle that had been in contact with the inoculated animals.

The immunity produced by the modified live virus was such that 79 per cent of the nonreactors in the controlled experiments resisted challenge with bovine virus three weeks after vaccination.

In the field trials, an antibody titer adequate for protection was produced in one third of the cattle susceptible initially. This adequate titer persisted during a six-month observation period. There also was an increase in antibody titer in cattle that were immune initially—about one third of the total vaccinated.

References

[1] Cunha, R. G., Baptista, J. A., Jr., Serrão, U. M., and Torturella, I.: El uso de los ratones lactantes en la avaluación de los anticuerpos contra el virus de la fiebre aftosa y su significación inmunológica. Gac. vet., *19*, (1957): 243–267.

[2] Cunha, R. G., and Eichhorn, E. A.: Studies on Rabbit-Adapted Foot-and-Mouth Disease Virus: I. Propagation and Pathogenicity. Am. J. Vet. Res., *19*, (Oct., 1958): In press.

[3] Gribanov, V.: Résultats de l'épreuve du vaccin antiaphteux VIEV préparé avec un virus adapté au lapin. Bull. Off. Internat. des Épizoot., *53*, (1955): 632–635.

[4] Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. A. R. C. Rep. Series 9, H.M. Stationery Off. (London), 1949.

[5] Huck, R. A., and Hudson, J. R.: Cited by Wilsdon, A. J.: Swine Fever. Vet. Rec., *70*, (1958): 3–13.

[6] Pay, T. W. F.: The Propagation of the Virus of Foot-and-Mouth Disease in Monolayer Cultures of Adult Bovine Tongue Epithelium. Proc. Roy. Soc. Med., *50*, (1957): 912–922.

[7] Skinner, H. H., Henderson, W. M., and Brooksby, J. B.: Use of Unweaned White Mice in Foot-and-Mouth Disease Research. Nature, *169*, (1952): 794.

[8] Waldmann, O., and Nagel, H. C.: Sobre el valor del virus murinizado para la preparación de vacunas contra la fiebre aftosa. Gac. vet., *15*, (1953): 3–9.

Attempts to Immunize Guinea Pigs with Flury Vaccine and Subsequent Challenge with Fixed Rabies Virus

C. HUYGELEN, Dr. Vet. Med., and J. MORTELMANS, Dr. Vet. Med.

Astrida, Ruanda-Urundi, Belgian Congo

GUINEA PIGS were inoculated with Flury anti-rabies vaccine, by Koprowski and Black,[2] and subsequently challenged with street virus. They described a potency test based on the immunity of these vaccinated animals against street virus.

The resistance of vaccinated guinea pigs to challenge with fixed rabies virus has been studied on a small scale by Bindrich,[1] who obtained a significative difference in mortality ratio between vaccinated and control animals after intramuscular injection of a strain* of fixed virus, emulsified in a buffered 10 per cent solution of rat testicle extract. However, serum from vaccinated guinea pigs was proved to contain fixed virus-neutralizing antibodies by serum-neutralization tests carried out by Koprowski and Black.[2]

To our knowledge, no other experiments have been performed to determine the resistance of guinea pigs vaccinated with Flury vaccine to subsequent challenge with fixed virus.

MATERIALS AND METHODS

Virus Strains.—In all experiments, we used a Flury strain having undergone its forty-fifth egg passage. A 40 per cent chicken embryo suspension was made in normal saline solution, to which penicillin and streptomycin were added. Serial tenfold dilutions were made of this 40 per cent suspension, so that successive 1:25 and 1:250 dilutions of chicken embryo were obtained.

As the source of fixed virus for challenge purposes, we used the 1,945th rabbit brain passage of a strain originating from the Pasteur Institute in Paris. The brain of a sheep, inoculated with this strain and killed *in extremis*, was divided into 100 parts and kept in a freezer at −15 C. Serial tenfold dilutions were made of this brain in normal saline solution before each challenge test; 0.2 ml. was used for the intracerebral inoculation of guinea pigs.

Animals.—Guinea pigs, weighing 350 to 450 Gm., were obtained from the breeding colony of the laboratory.

Titration of the vaccine was carried out in the usual way by intracerebral inoculation of serial tenfold dilutions in Swiss albino mice, obtained from the breeding colony of the laboratory. Each mouse was given 0.03 ml. of the respective dilutions and was observed for 21 days; 6 mice were used for each dilution.

EXPERIMENTAL PROCEDURE AND RESULTS

Determination of the l.d.$_{50}$ of the Strain of Fixed Rabies Virus for Guinea Pigs by the Intracerebral Route.—Three successive experiments were carried out to determine the l.d.$_{50}$ of the fixed-virus strain used. In the first experiment, 24 guinea pigs (4 lots of 6 guinea pigs each) were inoculated intracerebrally with 0.2 ml. of the sheep brain suspension in tenfold dilutions, ranging from 10^{-2} to 10^{-5} (table 1).

The first experiment demonstrated that higher dilutions were required for accurate determination of the l.d.$_{50}$. Therefore, in a second series of guinea pigs inoculated in the same way, higher dilutions were used, ranging from 10^{-4} to 10^{-7}. A third experiment confirmed the results of the two previous ones with a minor difference, inherent to every biological titration.

The l.d.$_{50}$ in experiment 2 was $10^{-5.8}$; in experiment 3, $10^{-5.5}$. The results were sufficiently constant to be considered as a basis for comparison in further experiments with vaccinated guinea pigs.

The number of days between inoculation and death depended on the dilution of the virus (table 2). A period of 21 days was fixed as the limit for the time of observation; the rare mortalities occurring after this could not influence the results significantly.

Vaccination of Guinea Pigs with Flury Vaccine.—Three groups of 120 guinea pigs each were inoculated, respectively,

TABLE 1—Mortality Ratio of Guinea Pigs After Intracerebral Inoculation of Fixed Virus

Dilution of fixed virus	Experiments		
	1	2	3
10^{-2}	5/5	.....	.....
10^{-3}	6/6	.....	.....
10^{-4}	4/5	6/6	6/6
10^{-5}	3/5	6/6	3/6
10^{-6}	.....	1/6	2/5
10^{-7}	.....	1/6	1/6

From the Veterinary Research Laboratory, Astrida, Ruanda-Urundi, Belgian Congo, Africa.

* Produced by Pitman-Moore Co., Indianapolis, Ind.

XUM

TABLE 2—Number of Days Between Inoculation of Fixed Virus and Death

Dilution of fixed virus	No. days																
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
10^{-2}	4*	1	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
10^{-3}	3	3	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
10^{-4}	1	12	3	....	....	....	....	....	....	....	....	....	....	....	....	....	....
10^{-5}	1	2	3	1	2	2	....	....	1	....	....	....	....	....	....	....	....
10^{-6}	....	....	....	....	....	....	....	....	....	....	....	1	1	1	....	....	....
10^{-7}	....	....	....	....	....	....	....	....	1	1	....	....	....	....	....	....	....

* Number of mortalities per day.

with 40.0 per cent, 4.0 per cent, and 0.04 per cent suspensions of chicken embryo tissue. Each guinea pig was given 1 ml. intramuscularly in the hindleg. The mortality ratios due to the Flury virus were high in the first two groups, whereas only a small number of deaths occurred (1.0 to 2.0%) in the third group. The animals of the two groups given 40.0 or 4.0 per cent suspensions could not be challenged with fixed virus during the first three weeks after inoculation, since it would have been impossible to determine whether the mortalities were due to the Flury vaccine or to the fixed virus. This would have led to misinterpretation of the results. More reliable results could be obtained only when the deaths due to the Flury virus had almost stopped, *i.e.*, after a period of three weeks following inoculation.

Challenge of Vaccinated Guinea Pigs with Intracerebral Inoculation of Fixed Virus.—On postinoculation days 8, 15, 22, and 31, a certain number of guinea pigs were taken from the group given 1 ml. of a 0.4 per cent suspension of Flury vaccine; these animals were inoculated by the intracerebral route with serial tenfold dilutions of fixed virus. Mortalities occurring from postinoculation day 5 to 21 were used to calculate the mortality ratio (table 3). The $l.d._{50}$, calculated according to the method of Reed and Muench, was $10^{-6.3}$ on day 8, $10^{-6.6}$ on day 15, $10^{-5.6}$ on day 22, and $10^{-4.3}$ on day 31.

After the challenge on days 8 and 15, higher mortality ratios were noticed than in unvaccinated guinea pigs. The cause of this phenomenon is not clear, but might be analogous to the negative immunity phase recorded in some other infections. On day 22, the $l.d._{50}$ returned to the level of the mortality ratio of the control animals and, on day 31, a slight decrease of the $l.d._{50}$ was noticed. The latter experiment, however, was carried out with a reduced number of animals (4 instead of 6 for each series), and a higher percentage of these died within the first days after the inoculation of fixed virus.

TABLE 3—Mortality Ratio of Guinea Pigs Following Challenge By Intracerebral Inoculation of Fixed Virus

Dilution of fixed virus	Postvaccination days			
	8	15	22	31
10^{-3}	6/6	6/6	3/3	3/3
10^{-4}	6/6	6/6	4/4	1/2
10^{-5}	5/6	6/6	4/5	1/4
10^{-6}	3/6	4/5	1/5	0/3
10^{-7}	3/6	2/5	1/4	.....

The number of animals which died within the first four days increased proportionally to the length of the period between vaccination and challenge: 0 of 30 after challenge on day 8, 2 of 30 on day 15, 9 of 30 on day 22, and 4 of 16 on day 31. This phenomenon may perhaps be attributed to an allergy provoked by the previous inoculation of Flury virus.

Another experiment with guinea pigs, vaccinated with a higher dose of Flury vaccine (1 ml. of a 4% chicken embryo suspension), confirmed that the immunity acquired by vaccinated guinea pigs against subsequent intracerebral challenge with fixed virus on day 31 was nonexistent or negligible. This experiment revealed an $l.d._{50}$ of $10^{-5.0}$ (all of 7 died at lesser dilutions; 0 of 3 at 10^{-6}). One fourth of these animals also died during the first four days after inoculation, probably because of allergic reactions.

Challenge of Vaccinated Guinea Pigs by Intramuscular Inoculation with Fixed Virus.—Ten guinea pigs, vaccinated with 1 ml. of a 4 per cent suspension of chicken embryo tissue, were challenged on postvaccination day 22 by the intramuscular route with 1 ml. of a 10 per cent dilution of fixed virus. Ten control animals were inoculated in the same way with fixed virus; in both instances, the injection was given in the masseter muscles. Two vaccinated guinea pigs died on postchallenge day 3; the remaining 8 and the 10 controls died between days 5 and 11.

XUM

TABLE 4—Challenge of Guinea Pigs, Vaccinated with Several Dilutions of Flury Vaccine, by Subsequent Intramuscular Injection of Several Dilutions of Fixed Virus

Dilution of fixed virus	Mortality ratio of guinea pigs vaccinated with Flury vaccine			Mortality ratio of control animals
	40.0%	4.0%	0.4%	
1:10	1/2	0/2	1/2	2/2
1:20	0/3	0/3	1/3	2/3
1:40	0/3	0/3	2/3	2/2
1:80	0/3	0/3	0/3	2/3

In an additional experiment, three groups of guinea pigs were given 1 ml. of a 40.0 per cent solution, 1 ml. of a 4.0 per cent solution, and 1 ml. of a 0.4 per cent solution, respectively. A fourth group served as controls. They were challenged on day 31 with 1 ml. of serial twofold dilutions of fixed virus, ranging from 1:10 to 1:80, in the masseter muscles (table 4).

The mortality ratio was much lower in the vaccinated guinea pigs than in the control animals, but the difference was less marked in the group given the 0.4 per cent suspension than in the groups vaccinated with higher concentrations of Flury vaccine.

Neutralization Tests.—Serum was taken from two groups of 4 guinea pigs each which had been vaccinated with a 40 per cent embryo suspension 30 days previously. Serial tenfold dilutions of fixed virus were mixed with equal volumes of this serum. The serums of the individuals within each group had been mixed previously, but individual titration of the antibody level of each guinea pig was not carried out, since it had been clearly demonstrated by Koprowski and Black [2] that no direct relationship exists between the antibody titer of an individual animal and its resistance to challenge with rabies virus. Guinea pigs showing a serum titer of 1:10 or lower, as well as those showing a titer of 1:655, seemed to be protected against challenge inoculation.

The neutralization tests demonstrated the presence of fixed virus-neutralizing antibodies. The $l.d._{50}$'s in the two groups were $10^{-2.5}$ and $10^{-3.7}$, respectively, whereas the $l.d._{50}$ in control animals was $10^{-5.6}$.

DISCUSSION

The aim of these experiments was to find a potency test for live-virus rabies vaccine based on a challenge inoculation of fixed virus. Street virus is too dangerous to be used in routine testing of rabies vaccine in laboratories not equipped for this purpose.

For the potency determination of fixed-virus vaccines (chiefly, phenolized vaccines), the Habel test is used in many countries. It has the advantage of being simple and safe and requires only a short time before results are obtained. However, this test is carried out in a way which is different from natural rabies infection, not only because fixed virus is used instead of street virus, but because the virus is administered intracerebrally.

Recently, Krause [3] studied another test using fixed virus by plantar instead of intracerebral inoculation. These experiments seem to give reliable results for the testing of different fixed-virus vaccines (Semple, Hempt); the mortality curves, instead of being biphasic or multiphasic, as observed in the Habel test, were all monophasic.

SUMMARY AND CONCLUSIONS

Guinea pigs, vaccinated by intramuscular injection of Flury vaccine, were challanged by the intracerebral route with a strain of fixed rabies virus on postvaccination days 8, 15, 22, and 31. Resistance to this challenge was either negligible or nonexistent.

Guinea pigs vaccinated in the same way, but challenged with fixed virus by the intramuscular route, appeared to have better protection.

Serum-neutralization tests proved the presence of fixed virus-neutralizing antibodies in the serum of vaccinated guinea pigs.

These experiments indicate that challenge by intracerebral inoculation of fixed virus is of no value in testing Flury vaccine in guinea pigs.

References

[1] Bindrich, H.: Untersuchungen über den "Flury"-Stamm des Tollwutvirus. II. Mitteilung: Immunisierungsversuche. Arch. f. exptl. Vet.-Med., *10*, (1956): 226–236.

[2] Koprowski, H., and Black, J.: Studies on Chick Embryo Adapted Rabies Virus. IV. Immunization of Guinea Pigs and Description of a Potency Control Test. J. Immunol., *72*, (1954): 79–84.

[3] Krause, W. W.: Kritische experimentelle Studien über die Prüfung von Tollwutimpfstoffen, die Pathogenese der Lyssa und das Geschehen in der Inkubationsperiode. I. Mitteilung: Die Prüfung der Tollwutimpfstoffe. Zentralbl. Bakt., *167*, (1957): 458–480.

XUM

Characterization of *Vibrio Fetus* Antigens
I. Chemical Properties and Serological Activities of a Soluble Antigen

MIODRAG RISTIC, Dipl.Vet., D.V.M., M.S., and C. A. BRANDLY, D.V.M., M.S.

Urbana, Illinois

ALTHOUGH the immunoserological features of *Vibrio fetus* have been studied in some detail, comparatively little work has been reported on the isolation and characterization of soluble antigens from the organism.

In an attempt to isolate a soluble substance from *V. fetus*, Gallut [2] used the method originally described by Palmer and Gerlough [11] with typhoid organisms. Gallut isolated two antigenic substances on the basis of *V. fetus* solubility in 95 per cent phenol. From the phenol-insoluble fraction, he isolated a water-soluble substance which reacted positively to the Molisch test and, therefore, was considered carbohydrate in nature. The phenol-soluble fraction gave a negative reaction to the Molisch test and a positive reaction to the biuret, xanthoproteic, and Millon tests. It, therefore, was considered to be proteinaceous in nature. When the two fractions were used in precipitation tests, the phenol-soluble substance was antigenically shared by most of the strains studied.

Gallut did not relate the newly described colonial variational phenomena of *V. fetus* to their usual concomitant antigenic changes.[13] Cultures from which soluble antigens were secured could have been in various stages of dissociation which eventually would reflect the qualitative and quantitative configuration of the antigen.

The over-all purpose of this study was the isolation of a soluble antigen from a smooth form of *V. fetus* cells to be used for detection of specific circulating antibodies.

This paper describes a simple method of extracting a heat-stable, water-soluble antigenic substance from the smooth cells of *V. fetus*. In addition to the conventional chemical procedures, chromatographic techniques and ultraviolet absorption spectra were used in the identification of the components of this antigenic fraction. Precipitation reaction in gels was one of several serological tests used in studies of antigenic reactivity of the substance.

From the Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana. This paper contains data included in a thesis to be submitted by the senior author to the University of Illinois in partial fulfillment of the requirements for the Ph.D. degree.

The study of the senior author was made possible by the grant of a fellowship by the American Veterinary Medical Association through its Research Council.

This investigation was supported in part by a research grant (E-1183) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

MATERIALS AND METHODS

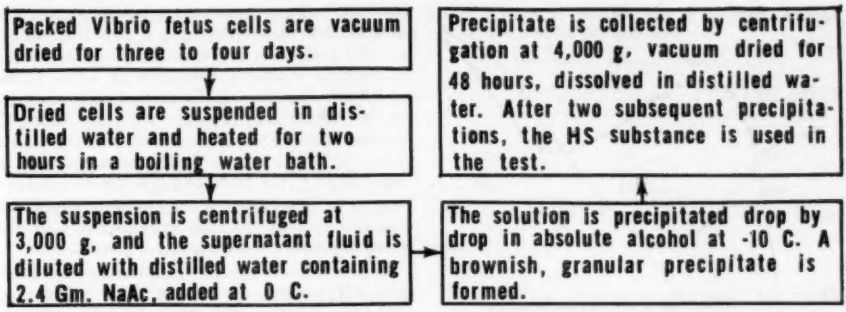
Preparation of Cells.—A smooth form of *V. fetus*, strain UF, originally isolated from the stomach content of an aborted fetus by the senior author in 1953, was the source of a heat-stable, water-soluble fraction (HS). To select a smooth form, the *V. fetus* culture was streaked on solid Brucella-Albimi * agar plates. To facilitate obtaining discrete colonies, 0.5 per cent agar was added, which resulted in a 2.0 per cent final agar concentration of the medium.

After incubation in an atmosphere of 45.0 per cent CO_2 for 72 hours at 37 C., the colonies were examined under x 45 magnification, using oblique, transmitted light according to the method of Henry.[8] Several transfers were made and smooth variants selected each time until growth was secured which represented a pure smooth colony population. The growth was washed off with 6.0 ml. of sterile 0.85 per cent sodium chloride solution, and 0.5 ml. of the resulting suspension was inoculated on Brucella-Albimi agar in 16-oz. prescription bottles. A loopful of the inoculum was streaked on a duplicate Brucella-Albimi agar plate, which served as a control of the smooth variant stability.

The cultures were incubated in the manner described previously. After incubation, cultures were removed from the agar surface with sterile 0.85 per cent sodium chloride solution and washed by means of three successive centrifugations at 2,500 g. The packed cells were stored at 4 C. until a desired amount could be harvested. The packed cells were vacuum dried at room temperature for four days, after which time they were weighed.

Isolation of Fraction.—The fractionation procedure is outlined (graph 1). To 2.216 Gm. of dried cells, 25 ml. of distilled water, pH 7, was added and the suspension was immersed in boiling water for two hours. The material was centrifuged at 3,000 g and the resulting yellowish brown supernatant fluid was collected and stored at 4 C. The sediment was similarly heat treated two additional times. This was followed by collection and separate preservation. To each lot of the supernatant fluid, 2.4 Gm. of sodium acetate in 10 ml. of distilled water was added at 0 C. Each solution was poured drop by drop into 2 volumes of absolute ethyl

* A product of Albimi Laboratories, Brooklyn, N.Y.


Packed Vibrio fetus cells are vacuum dried for three to four days.
Dried cells are suspended in distilled water and heated for two hours in a boiling water bath.
The suspension is centrifuged at 3,000 g, and the supernatant fluid is diluted with distilled water containing 2.4 Gm. NaAc, added at 0 C.
The solution is precipitated drop by drop in absolute alcohol at -10 C. A brownish, granular precipitate is formed.
Precipitate is collected by centrifugation at 4,000 g, vacuum dried for 48 hours, dissolved in distilled water. After two subsequent precipitations, the HS substance is used in the test.

Graph 1—The isolation of heat-stable (HS) soluble antigen from the smooth form of Vibrio fetus, strain UF.

alcohol which was cooled to −10 C. A brownish granular precipitate was formed immediately. The precipitate was collected by centrifugation at 4,000 g for 15 minutes. The sediment was vacuum dried for 48 hours, after which it was dissolved in 30 ml. of distilled water. Further purification of the fraction was accomplished by two successive precipitations. Ten milligrams of the fraction HS was added to 2 ml. of distilled water, and the material was stored at −20 C. until used.

Analytical Procedure.—Total solids were determined by drying aliquots in vacuum at room temperature to a constant weight. The biuret, Millon, xanthoproteic, ninhydrin, and Molisch tests were used to determine the general nature of nonhydrolyzed specimens of the substance. Total nitrogen determinations were accomplished by a micro-Kjeldahl procedure, using K_2SO_4-$CuSO_4$ as the digestion catalyst. Total carbohydrates were determined colorimetrically with anthrone reagent according to the method of Morris.[9] Total pentose was determined with orcinol-ferric chloride reagent.[14] For phosphorus determination, the sample was digested with 10 N H_2SO_4 in the oven for 30 to 60 minutes at 130 to 160 C., and the total inorganic phosphorus was determined by means of Fiske-Subbrow tests.[14]

The paper chromatography technique originally introduced by Martin and Synge[5] was used for identification of the reducing sugars and amino acids liberated by acid hydrolysis of the fraction. The following solvents were used: water-saturated phenol, water-saturated phenol in an atmosphere of ammonia, water-saturated phenol-acetic acid, and *n*-butanol acetic acid-water. Silver nitrate, triphenyltetrazolium chloride, aniline hydrogen phthalate, and Bial's orcinol reagent were used as color developing agents in the identification of reducing sugars. Detection of amino acids on chromatograms was accomplished, using 0.25 per cent ninhydrin reagent in acetone.

To determine pH stability of the substance, M-5 acetate buffers were used for the pH values from 3.0 to 6.0, while M-5 phosphate buffers were used for the pH values from 6.0 to 11.0. Ultraviolet absorption spectra were measured on the Beckman DU spectrophotometer. Bovine serum was used as the comparative protein-containing sample.

Serological Methods—Preparation of Antiserum Against HS Fraction.—Animals were inoculated with a series of four injections at four-day intervals. Each inoculum consisted of 1 mg. of the HS fraction in 1 ml. of 0.85 per cent sodium chloride solution. One rabbit was inoculated intravenously, 1 rabbit intramuscularly, and 1 guinea pig intramuscularly. The animals were exsanguinated by cardiac puncture five days after the final injection. Four hours after collection, the serum was removed

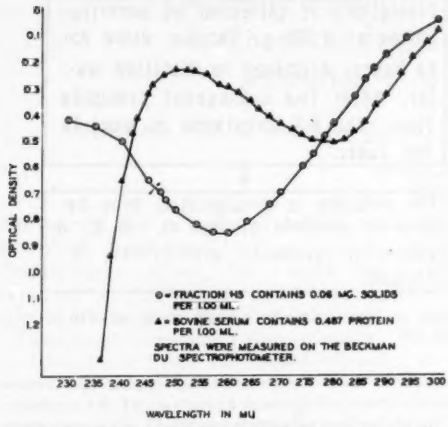
TABLE 1—Yield and Composition of Fraction HS of Smooth Form of Vibrio Fetus, Strain UF

Dry weight of cells	Hours of boiling	Yield of solids	Total nitrogen	Total carbohydrate	Total phosphorus
2.216 Gm.	2	0.325 Gm.			
	4	0.043 Gm.			
	6	0.030 Gm.	13%	14%	6.5%
		Total, 0.398 Gm. (17.9%)			

Carbohydrate differential		Amino acids differential	
Pentoses (ribose)	14%	(1) Lysine	(4) Valine
Hexoses	0%	(2) Arginine	(5) Leucine
		(3) Threonine	(6) Serine
		(7) Alanine	

Total solids were determined by drying aliquots to constant weight in vacuum. Determinations of nitrogen were done by the micro-Kjeldahl test; of total carbohydate, by anthron reagent; pentoses, by the Meijbaum test; phosphorus by the Fiske-Subbrow test. Carbohydrate and amino acid differentials were done by paper chromatography.

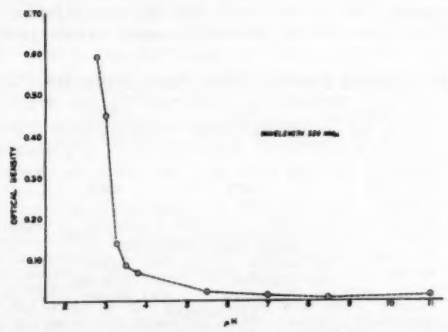
XUM



Graph 2—The ultraviolet absorption spectra of the heat-stable fraction (HS) and normal bovine serum.

and stored at −20 C. in a sealed container in 1-ml. amounts.

Agglutination Test.—Antigens were prepared by growing the organisms on Brucella-Albimi agar (2.0% agar) in 16-oz. prescription bottles. The bottles were incubated as described previously. Formolized antigens were prepared by suspending the cells in 0.3 per cent formaldehyde solution containing 0.85 per cent sodium chloride. Heat-treated antigens were prepared by boiling washed cells for two hours, washing them twice by centrifugation and, finally, resuspending the cells in 0.3 per cent formaldehyde-saline solution. The concentrated antigens were stored in the refrigerator. Prior to use, the antigens were standardized to a density of approximately 55.0 per cent light transmission of the suspensions in formaldehyde-saline solution, using a Leitz photoelectric colorimeter with a 550 mμ filter. This density approximated tube 3 of the MacFarland nephelometer. Agglutination tests were conducted by preparing a 1:5 dilution of the specific antiserum in formaldehyde-saline solution. Subsequent dilutions consisted of serial transfers of 0.5-ml. amounts of the diluted antiserum in 0.5 ml. of formaldehyde-saline solution. Antigen in the amount of 0.5 ml. was added to make final dilutions (1:10, 1:20, etc.). The mixtures were incubated at 37 C. for 40 to 48 hours before results were recorded. The criterion for complete agglutination was evidence of clearing with presence of clumps when the tubes were agitated gently.



Graph 3—The stability of the heat-stable fraction (HS) at various pH values. One milligram of HS in 5.0 ml. of a buffer was used for each point plotted.

Inhibition of Specific Agglutination by HS Fraction.—Hyperimmune serums from rabbits which had been injected with whole *V. fetus* variant cultures and whose serums agglutinated homologous antigens to high titers were selected for this purpose.[18] For the inhibition test, a serum dilution near the end point of agglutination was selected; it contained four times the concentration of serum of the highest tube which, after two hours' incubation at 56 C. in a water bath, consistently gave a definite agglutination reaction with homologous whole cell preparations. Five tenths milliliter of the selected antiserum dilution was added to a series of tubes containing 0.5 ml. of serial dilutions of the HS substance, to be tested for its ability to inhibit the specific agglutination. After two hours' incubation at 56 C. in a water bath, 1.0 ml. of the whole cell antigen suspension was added to each tube and the tests were incubated for an additional two hours. Appropriate control tubes in which saline solution was substituted for HS substance were included in the test.

Precipitation Reaction in Gel.—The double diffusion technique of Ouchterlony[30] was used; in this technique, zones of precipitate are formed when antibody and antigen are allowed to interdiffuse in a thin layer of agar. An agar layer, approximately 1 mm. in thickness, was prepared in a Petri dish and allowed to dry and harden. A metal template, with three ⅜-sq.-inch legs attached to the upper metal support plate, was placed in a dish. A layer, approximately 3 mm. thick, of 2.0 per cent melted Bacto agar containing 0.85 per cent NaCl and 0.5 per cent phenol was poured into the dish over the basic agar layer and allowed to harden. When the template was removed, the depressions remained in the agar, one of which was filled with approximately 0.3 ml. of 10 mg./100 ml. HS fraction, while the other two openings were filled with the antiserums being examined. The plates were left at room temperature and the zones of precipitate observed and recorded after four days.

RESULTS

Purified fraction HS reacted positively to the Molisch test for carbohydrates and negatively to the biuret, Millon, xanthoproteic, and ninhydrin tests for proteins. Data are presented on yield and chemical composition of the soluble fraction HS (table 1).

TABLE 2—Agglutination Reaction Between Three Types of Antiserums Produced Against Fraction HS and Variants of Vibrio Fetus, Strain UF

Strain	Variant	Antigen form	Antiserums against fraction HS		
			Rabbits injected		Guinea pigs injected
			Intravenously	Intramuscularly	Intramuscularly
UF	Smooth	Formolized *	640 **	80	40
	Smooth	Heated †	5,120	160	20
	Rough	Formolized	40	80	40
	Rough	Heated	80	40	40

* Suspended in 0.3 per cent formaldehyde-saline solution; † heated for two hours in boiling water; ** titer expressed as reciprocal of serum dilution.
Fraction HS was secured from smooth variant of strain UF.

From 2.216 Gm. of dried cells, a total of 0.398 Gm. (or 17.9%) of purified fraction was obtained. The first two hours of heat treatment yielded 0.312 Gm., while a total of 0.073 Gm. of the fraction resulted from the subsequent four-hour treatment. Total nitrogen content was 13.0 per cent. Total carbohydrate, calculated as glucose, was 14.0 per cent. After acid hydrolysis, total carbohydrate remained the same as in unhydrolyzed material. All of the carbohydrate was represented by pentoses as determined with orcinol-ferric chloride reagent. The total content of inorganic phosphorus was 6.5 per cent. The paper chromatographic method demonstrated the presence of the following amino acids: lysine, arginine, threonine, valine, leucine, serine, and alanine.

Individual amino acids were identified by simultaneously employing specimens of known amino acids and determining comparatively the Rf values, using various solvents. Fraction HS exhibited maximal ultraviolet absorption at 257 to 260 mμ (graph 2).

The solutions containing HS fraction generally were neutral or slightly acid in reaction. The fraction showed no buffering capacity. The solutions of the fraction remained stable when mixed with M-5 phosphate buffer throughout the pH range of 6.0 to 11.0. When the serial pH values from 6.0 to 3.0 were prepared by use of M-5 acetate buffer, progressive increase of cloudiness resulted until flocculation occurred at pH 3.5, with maximal reaction at pH 3.0 (graph 3). In the absence of salts, the addition of acids (acetic or hydrochloric) produced increase of turbidity with flocculation at pH 3.5. When a trace of alkali was added to restore a neutral reaction, the precipitate was dissolved completely.

The antiserum obtained from a rabbit following intravenous injection of HS fraction revealed the presence of antibody capable of agglutinating the cells of *V. fetus*, strain UF, as follows: smooth formolized cells and smooth heated cells were agglutinated in serum dilutions of 1:640 and 1:5,120, respectively (table 2). Rough formolized and rough heated cells were agglutinated in dilutions of 1:40 and 1:80, respectively. The antiserum resulting from intramuscular injections reacted in considerably lower dilutions with several antigens

TABLE 3—Inhibition of Specific Vibrio Fetus Agglutination Reactions by HS Fraction of Strain UF

Antigen source	Antigen form	Specific *V. fetus* antiserums: Homologous agglutination titer	Greatest dilution of HS fraction which inhibited agglutination
UF, bovine	Smooth, formolized †	5,120	1:64 *
	Smooth, heated **	5,120	1:128
	Rough, formolized	20,480	1:512
	Rough, heated	5,120	1:512
Ill., ovine	Smooth, formolized	1,280	1:16
	Smooth, heated	2,560	1:128
	Rough,formolized	1,280	1:1,024
	Rough, heated	5,120	1:512
1134, human	Smooth, formolized	1,280	
	Smooth, heated	2,560	No inhibition
	Rough, formolized	640	
	Rough, heated	320	

* Original concentration of fraction HS from which dilutions were made was 1 mg./1.00 ml. of saline solution; † suspended in 0.3 per cent formaldehyde-saline solution; ** heated for two hours in boiling water.

XUM

of UF strain than did that secured after intravenous injection. The maximum agglutination titer obtained with antiserum produced by intramuscular injections of guinea pigs was 1:40.

Serial dilutions of fraction HS were made in saline solution and tested for their ability to inhibit the specific agglutination. Results of this test are summarized (table 3). Various concentrations of fraction HS were capable of blocking agglutination of the variants of the homologous strain UF of bovine origin, as well as within the strain Ill. of ovine origin. There was no inhibition of agglutination reaction of strain 1134 of human origin. The greatest amount of the fraction needed to inhibit the agglutination reaction was 0.125 mg., in the case of smooth formolized variant of strain Ill., while the least amount required was 0.0010 mg., in the case of rough formolized variant of strain Ill.

The results of precipitation reactions in gels are presented (table 4). Precipitation

TABLE 4—Precipitation Reaction in Gel Between Fraction HS of Smooth Vibrio Fetus, Strain UF, and Specific Antiserums of Homologous Variant Antigens

	Specific antiserum against fraction HS (0.1 mg./1.0 ml.)		
Strain	Variant	Antigen form	Precipitation reactions
UF	Smooth	Formolized *	(+) (−)
	Smooth	Heated †	(+) (±)
	Rough	Formolized	(+) (−) (±)
	Rough	Heated	(+) (−)

* Suspended in 0.3 per cent formaldehyde-saline solution; † heated for two hours in boiling water.
(+) = reaction of identity; (±) = reaction of partial identity; and (−) = reaction of nonidentity.

of identity was observed with each of the four variant antiserums. Precipitation of partial identity was observed with antiserums of smooth heated and rough formolized variant cells. Precipitation of no identity was shown by antiserums of smooth formolized cells and rough variant cells.

DISCUSSION

This study has been directed toward development of methods of isolation and identification of an antigenic substance common to *V. fetus* strains. Although the amount of HS fraction needed to inhibit specific agglutination reactions varied with the variant strain used, the results indicated that the fraction is antigenically shared by strains UF and Ill., of bovine and ovine origin, respectively. It was established by Wiidik and Hlidar [16] and Ristic *et al.*[13] that serogroups of *V. fetus* contain, in addition to flagellar H antigens, somatic thermostable O antigens and thermolabile superficial or envelope antigens. In our study, the antiserum against fraction HS contained agglutinating antibody against both, *i.e.*, of heated antigens, from which cells a heat-labile portion was removed, and formolized antigens, represented by intact cells with their heat-labile cell layers present. In view of this fact, the HS fraction appears to be a heat-stable component of the deeper structure of the cell occuring also within the heat-labile superficial portion of the *V. fetus* body.[13] It is generally understood that dominant or major antigen or antigens are primarily manifested in bacterial agglutination tests. The fact that the free substance, HS, was capable of inhibiting whole cell agglutination reaction characterizes it as being more than a secondary antigenic component of the cell.

A number of workers have freed a polysaccharide-protein complex from gram-negative bacteria.[6-8, 11, 15] These bacterial components represented O-specific antigens in which the polysaccharide part was a determinant of specificity, while the protein portion generally contributed to increased antigenicity. More recent work [4] has shown that a complex hapten can produce antibodies in mice, horses, and human beings. Fraction HS was capable of producing the antibody and reacting specifically with such an antibody. However, the tests used failed to demonstrate the presence of protein in the fraction, making it difficult to distinguish between a complete antigen and complex hapten with respect to the antigenic identity of HS fraction. The presence of seven amino acids (lysine, arginine, threonine, valine, leucine, serine, and alanine) may represent protein-like material similar to that observed by Miles and Pirie [6] in extracts from Brucella species. Such material would contribute increasingly to the antigenicity of HS fraction.

The nucleoproteins are known to absorb the maximum ultraviolet spectra at a wave length of 260 mμ, while proteins exhibit this characteristic at a wave length of 280 mμ. Fraction HS demonstrated an ultraviolet absorption peak at 257 to 260 mμ, which would indicate the presence of nucleoprotein or fractions of nucleoprotein. In agreement with this finding are the phosphorus content and the presence of ribose.

Purines and pyrimidines, however, were not detected by paper chromatographic methods with any of the stains used. It should be mentioned, nevertheless, that unstained areas corresponding with Rf values of purines and pyrimidines were observed when filter paper containing hydrolyzed fraction HS was exposed to ultraviolet light in a dark room. The presence of immunologically reactive nucleoproteins and serologically active pentoses associated with polysaccharides have been described as bacterial components.[1] It should be pointed out, however, that the HS substance failed to meet the criteria characterizing proteins, *i.e.*, (1) it failed to give positive reactions with the ninhydrin, Millon, xanthroproteic, and biuret tests; (2) it migrated easily in chromatogram paper; (3) it exhibited great heat stability without denaturation of its antigenic behavior.

The method of isolation of the soluble fraction may be improved by decreasing the boiling exposure time, in which case danger of degrading native cellular constituents will be minimized. In order not to damage possible thermolabile serologically active antigen, or antigens, inherent in the living *V. fetus* cell, the isolation by heat could be initiated and accomplished by enzyme digestion or treatment of other kind. In preliminary studies, a water-soluble protein-polysaccharide substance was obtained from *V. fetus* by combined treatment of cells with phenol and heat for ten to 15 minutes.

Antigenic heterogeneity among *V. fetus* strains and colonial dissociation with concomitant antigenic variation within the single strain makes selection of a reliable test antigen more difficult and time consuming. In view of this fact, additional studies are needed, with reference to the isolation of an antigen common to all strains of *V. fetus* and restricted to this species.

SUMMARY

A heat-stable, water-soluble substance, termed "HS," apparently partly polysaccharide in nature, was isolated from smooth *Vibrio fetus* cells of bovine origin. Its carbohydrates yielded only pentoses on hydrolysis. Seven amino acids were identified in the fraction. The fraction contained 14.0 per cent nitrogen and 6.5 per cent phosphorus. Immunological reactivity of the fraction was demonstrated by production of agglutinating antibody in the rabbit and guinea pig. Fraction HS was serologically active in gel precipitation tests and was capable of inhibiting a specific agglutination reaction.

References

[1] Dubos, R. J.: The Bacterial Cell. Harvard University Press, Cambridge, Mass. (1945): 113.

[2] Gallut, J.: Recherches Immunochémiques sur *Vibrio fetus*. I. Étude Sérologique Préliminaire de 10 Souches d'Origines Diverses. Ann. Inst. Pasteur, *83*, (1952): 449–454.

[3] Henry, B.: Dissociation in the Genus Brucella. J. Infect. Dis., *52*, (1933): 374–402.

[4] Landsteiner, K.: The Specificity of Serological Reactions. Revised ed. Harvard University Press, Cambridge, Mass., 1947.

[5] Martin, J. P., and Synge, L. M.: A New Form of Chromatograms Employing Two Liquid Phases. I. A Theory of Chromatography. II. Application to the Micro-Determination of the Higher Monoamino Acids in Proteins. Biochem. J., *35*, (1941): 1358–1364.

[6] Miles, A. A., and Pirie, N. W.: The Properties of Antigenic Preparations from *Brucella Melitensis*. I. Chemical and Physical Properties of Bacterial Fractions. Brit. J. Exptl. Path., *20*, (1939): 83–98.

[7] Miles, A. A., and Pirie, N. W.: The Properties of Antigenic Preparations from *Brucella Melitensis*. II. Serological Properties of the Antigens. Brit. J. Exptl. Path., *20*, (1939): 109–121.

[8] Morgan, W. T. J., and Partridge, S. M.: Studies in Immunochemistry. VI. The Use of Phenol and of Alkali in the Degradation of Antigenic Material Isolated from *Bacteria Dysenteriae* (Shiga). Biochem. J., *35*, (1941): 1140–1163.

[9] Morris, D. L.: Quantitative Determination of Carbohydrates with Dreywood's Anthzone Reagent. Science, *107*, (1948): 254–255.

[10] Ouchterlony, O.: Antigen-Antibody Reactions in Gels and the Practical Application of This Phenomenon in the Laboratory Diagnosis of Diphtheria. M.D. thesis, Caroline Institute, Medical School, Stockholm, Sweden, 1949.

[11] Palmer, J. W., and Gerlough, T. D.: A Simple Method for Preparing Antigenic Substances from the Typhoid Bacillus. Science, *92*, (1940): 155–156.

[12] Ristic, M., Herzberg, M., and Sanders, D. A.: Colonial and Antigenic Variations of *Vibrio Fetus*. Am. J. Vet. Res., *17*, (Oct., 1956): 803–809.

[13] Ristic, M., White, F. H., Doty, R. B., Herzberg, M., and Sanders, D. A.: The Characteristics of Agglutinating Antigens of *Vibrio Fetus* Variants. I. Effects of Heat and Formalin on Serological Activity. Am. J. Vet. Res., *18*, (Oct., 1957): 764–770.

[14] Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis, Minn., 1954.

[15] Westphal, O., Lüderitz, O., and Biester, F.: Über die Extraktion von Bakterien mit Phenol-Wasser. Zschr. Naturforsch., *7b*, (1952): 148–155.

[16] Wiidik, R. W., and Hlidar, G. E.: Untersuchungen über die antigene Struktur von *Vibrio fetus* von Rind. Das Kapsel-oder K-Antigen von *Vibrio fetus*. Zentralbl. f. Vet.-Med., *2*, (1955): 238–250.

Characterization of *Vibrio Fetus* Antigens. II. Agglutination of Polysaccharide-Sensitized Sheep Erythrocytes by Specific Antiserums

MIODRAG RISTIC, Dipl.Vet., D.V.M., M.S., and C. A. BRANDLY, D.V.M., M.S.

Urbana, Illinois

THE EXISTENCE *in vivo* of at least two colonial types with corresponding cellular and antigenic characteristics has been shown among *Vibrio fetus* species of bovine origin.[20] On the basis of cross-agglutination reactions, strain specificity was shown by smooth types while cross reaction was manifested among mucoid and rough variants of homologous and heterologous strains.[2, 19, 21] In addition, thermostable antigens of *V. fetus* variants were serologically shared with hydrogen sulfide-positive vibrios isolated in mucoid cut-glass colony forms.[21] Under these circumstances, the bacterial agglutination test does not appear satisfactory as a means of detecting specific circulating antibodies against *V. fetus*, nor is it of practical use in identifying cultures isolated from animals with suspected infections.

It is established that certain bacterial antigens may be adsorbed on erythrocytes, thus rendering these erythrocytes specifically agglutinable by the serum antibody directed against the substances adsorbed.[1, 4, 8] While specific pretreatment of erythrocytes with tannic acid is necessary to make adsorption of protein antigens possible, the polysaccharide antigens are adsorbed on the intact washed erythrocytes. The soluble *V. fetus* antigen, "HS," the isolation and chemical nature of which were considered in the preceding paper,[18] was found adsorbable on intact washed sheep erythrocytes, rendering these cells agglutinable by specific blood serum antibodies.

This report describes observations on the agglutination of erythrocytes, sensitized with HS antigen, by the anti-*V. fetus* serums produced by injection of rabbits and those collected from naturally infected bulls. In addition, specific inhibition of hemagglutination reaction is reported.

MATERIALS AND METHODS

Vibrio fetus strains used in this study (table 1) were isolated from one of the following sources: stomach contents of aborted fetuses, vaginal and cervical mucosa of cattle and sheep, and blood of human beings in which a subacute bacterial endocarditis was diagnosed.[10] Unless indicated differently, only smooth forms of *V. fetus* strains were used for production of antiserums and HS fractions. The methods used for isolation and propagation of organisms and for securing desired variants have been described elsewhere.[19, 22] Preparation, procedure, and materials used in securing the erythrocyte-sensitizing fraction HS have been described in the preceding paper.[18] Heat-stable fraction (HS) has been prepared from strains UF, Ill., 1134, 4220, and 6867. Strains of UF, Ill., and 1134 were selected as representative of *V. fetus* on the basis of their cultural, morphological, and biochemical

TABLE 1—Origin and Biochemical Characteristics of Vibrio Fetus and Related Vibrio Strains

Origin	Strain	Catalase production	H_2S production
Bovine aborted fetus	UF	+	−
	B-1	+	−
	B-4	+	−
	B-6	+	−
	B-10	+	−
Bovine cervical mucus (with previous abortion)	F	+	−
Bovine vaginal mucus	4220	−	+
Ovine aborted fetus	Ill.	+	−
	53-D	+	−
Ovine vaginal mucus	6867	−	+
Human blood	1134	+	−
	1154	+	−
	1307	+	−

From the Department of Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana. This paper contains data included in a thesis to be submitted by the senior author to the University of Illinois in partial fulfillment of the requirements for the Ph.D. degree.

The study of the senior author was made possible by the grant of a fellowship by the American Veterinary Medical Association through its Research Council.

This investigation was supported in part by a research grant (E-1183) from the National Institute of Allergy and Infectious Disease, U. S. Public Health Service.

The authors thank Drs. R. B. Lank, Louisiana State University; W. N. Plastridge, University of Connecticut; A. H. Frank, Agricultural Research Service, U.S.D.A.; K. E. Price, Chas. Pfizer Co.; L. R. DiLiello, Maryland Livestock Sanitary Service; Miss E. O. King, U.S. Public Health Service; and Messrs. B. D. Firehammer, Montana Experiment Station, and H. E. Rhoades, University of Illinois, for supplying some of the reagents used in this study.

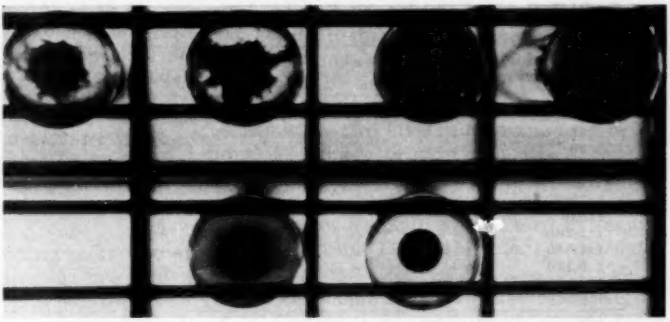


Fig. 1—Appearance of patterns of hemagglutination. Top row reactions (left to right): ++++, +++, ++, and +; bottom row: − and −.

characteristics and were isolated from bovine, ovine, and human sources, respectively. Vibrio strains 4220 and 6867 were noncatalase-producing organisms and originated from cattle and sheep, respectively.

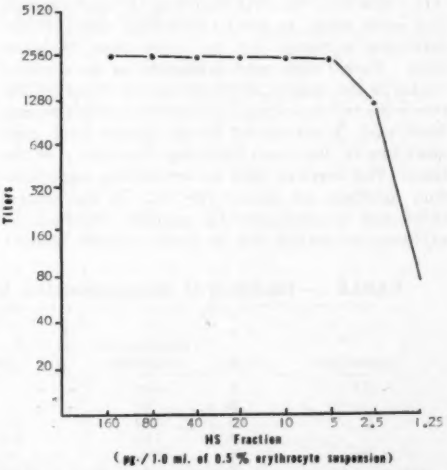
Production of Antiserum.—Antiserums were produced by rabbits inoculated intravenously, four times during a 21-day period, with approximate dosages of 10^7 cells suspended in 0.85 per cent sodium chloride solution. Prior to use, cultures were suspended at 4 C. in 0.3 per cent formaldehyde solution containing 0.85 per cent sodium chloride for at least 24 hours. The method used in preparing anti-HS serums has been described in the preceding paper.[18] Rabbits were exsanguinated by cardiac puncture and, after syneresis at 4 C. for two hours, the serum was collected and heated at 56 C. for 30 minutes to inactivate the complement.

In addition, specific serums representative of five serogroups [17] and serums representative of two serogroups [9, 15] were used. Twelve samples of serum from bulls, 9 of which were infected with *V. fetus*,[12] were preserved at 4 C. Merthiolate was added to make a final dilution of 1:10,000.

Preparation of Washed Sheep Erythrocytes.—Sheep blood was collected aseptically in an equal volume of sterile, modified Alsever's solution.[5] The erythrocytes in this mixture remain satisfactory for the hemagglutination test for two months when kept under aseptic conditions at 4 C. The mixture of blood and Alsever's solution was centrifuged and the erythrocytes were washed three times with 0.86 per cent sodium chloride solution. After the last centrifugation at 2,500 r.p.m. for 15 minutes in an International centrifuge (model V, size 2), the supernatant fluid was removed and the packed cells were stored at 4. C These washed cells were not used for hemagglutination if they were more than 5 days old or if they showed excessive hemolysis before that time.

Adsorption of Serum with Untreated Sheep Erythrocytes.—The purpose of this operation was to remove from the serums antibodies, unrelated to *V. fetus*, which would agglutinate untreated sheep erythrocytes. To 5.0 ml. of a 1:10 dilution of heated serum, 0.5 ml. of packed, washed, untreated erythrocytes was added and the suspension was incubated in a water bath at 37 C. for ten minutes. The suspension then was centrifuged and the supernatant fluid removed from sedimented cells and treated again in the same way with another 0.5 ml. of packed, untreated cells. The supernatant fluid was used in the hemagglutination test as a 1:10 dilution of adsorbed serum.

Sensitization of Erythrocytes with Fraction HS.—Packed erythrocytes, in an amount sufficient to give a 2.5 per cent final concentration of the suspension, were added to the desired volume of saline solution containing the HS fraction of known concentration. The mixture was placed in a water bath at 37 C. and left for two hours, with occasional agitation. After incubation, the cell suspension was centrifuged and the cells were washed three times with 15 times their volume of 0.85 per cent sodium chloride solution to remove excess HS substance. For use in hemagglutination and hemagglutination-inhibition tests, the cells were diluted



Graph 1—The effect of quantity of sensitizing antigen HS of Vibrio fetus, strain UF, on the hemagglutination titer. Serum was prepared against the whole cells of strain UF.

TABLE 2—Agglutination by Specific Antiserums of Sheep Erythrocytes Sensitized with Fraction HS of Vibrio Fetus, Strain UF

Dilutions of serum in saline solution	Antiserums							
	UF	B-6	B-10	F	B-4	B-1	Ill.	53-D
1:20	++++ *	++++	++++	++++	−	−	++++	++++
1:40	++++	++++	++++	++++	−	−	++++	++++
1:80	++++	+++	++++	++++	−	−	++++	++++
1:160	++++	++	++++	++++	−	−	++++	++++
1:320	++++	+	++++	++++	−	−	++++	++++
1:640	++++	−	++++	++++	−	−	++++	+++
1:1,280	++++	−	++++	+++	−	−	+++	+
1:2,560	++++	−	++++	+	−	−	+	−
1:5,120	++	−	+++	+	−	−	+	−
1:10,000	++	−	++	−	−	−	−	−
1:20,000	+	−	+	−	−	−	−	−

* Degree of agglutination.
Antiserums were produced againts smooth cells of the strains.

with 0.85 per cent sodium chloride solution to make a 0.5 per cent suspension.

Titration of HS Fraction and Hemagglutination Tests.—Fraction HS was titrated by sensitizing 10.0 ml. of 2.5 per cent erythrocyte suspension with varying amounts of the fraction. The greatest concentration of HS fraction used per 1.0 ml. of 0.5 per cent cells was 160.0 μg., while the least amount was 1.25 μg. Serial twofold dilutions of the adsorbed specific serum were prepared in saline solution to give a final volume of 0.5 ml., and 0.5 ml. of sensitized cell suspension was added to each tube. Two control tubes were used. One contained 0.5 ml. of saline solution and 0.5 ml. of 0.5 per cent suspension of treated cells, and the other contained 0.5 ml. of a 1:10 dilution of normal adsorbed serum and 0.5 ml. of 0.5 per cent suspension of treated cells.

The tubes were shaken well and left for approximately two and one half hours at room temperature. After this time, the first recording of agglutination was made, while the second recording was made the following morning, but not more than 18 hours later. There were only negligible or no discrepancies in the degree of agglutination observed between the two recording periods when rabbit serums were used. Reactions of bovine serums were most clear two to five hours following the setting of the tests. The criteria used in evaluating agglutination reactions are shown (fig. 1). In the control tubes and in tubes showing negative readings, the erythrocytes settled out in small, central, sharply demarcated discs, occasionally with narrow, hollow centers, which slipped from position when the tubes were tilted and which exhibited a homogeneous cloudy suspension upon more vigorous agitation. In strongly positive (4+) reactions, the erythrocytes settled out as compact granular agglutinates. The other reactions were as follows: 3+, a mat with a serrated margin was formed on the bottom of the tube; 2+, a smooth mat completely covering the bottom of the tube; and 1+, a large red ring with a finely serrated margin, which occasionally had a hollow center in an otherwise smooth bottom mat.

The Minimal Sensitizing Dose of Fraction HS.—The minimal sensitizing dose (m.s.d.) of fraction HS was considered to be the least amount of the fraction, in micrograms, required to sensitize 1.0 ml. of a 0.5 per cent erythrocyte suspension so that complete hemagglutination occurred at the maximal titer of the serum. Twice the m.s.d. was used in all tests.

Hemagglutination-Inhibition Tests.—The specificity of the hemagglutination test was ascertained by the ability of the free HS substance to inhibit specific hemagglutination. For this purpose, serums were selected which agglutinated the treated erythrocytes, and the serum dilution near the end point which consistently gave definite agglutination of treated cells was determined. Quantities of 0.2 ml. of these concentrations of selected antiserums were incubated in a 37 C. water bath for two hours with 0.2 ml. of graded dilutions of HS substance.

TABLE 3—Inhibition of Hemagglutination by Free Fraction HS of Vibrio Fetus, Strain UF

Antiserums	Type	Dilution of antiserum	Micrograms of fraction HS per 0.4 ml. of treated sheep erythrocytes										
			400	200	100	50	25	12	6	3	1.5	0.75	0
UF	S	640	−	−	−	−	−	−	−	2	1	1	4
	R	80	−	−	−	−	−	−	4	4	4	4	3
F	S	320	−	−	−	−	−	−	3	2	1	1	4
	R	160	−	−	−	−	−	2	1	1	2	1	4
Ill.	S	640	−	−	−	−	−	−	−	−	−	1	4
	R	40	−	−	−	−	−	−	−	2	3	3	3
UF	HS	2,560	−	−	−	−	−	−	−	−	−	2	3
Ill.	HS	1,280	−	−	−	−	−	3	3	2	2	2	4

S = smooth cells; R = rough cells; HS = heat-stable fraction of strain shown; 4 = ++++; 3 = +++; 2 = ++; 1 = + (agglutination).

XUM

To each tube was then added 0.4 ml. of 0.5 per cent suspension of treated erythrocytes. Incubation and the reading of results were carried out as in the case of the hemagglutination technique. The following control tubes were used: (1) fraction HS, antiserum, and untreated erythrocytes; (2) fraction HS, normal serum, and treated erythrocytes; and (3) saline solution substituted for HS fraction, antiserum, and treated erythrocytes.

RESULTS

The results on sensitizing activity of varying amounts of fraction HS are shown (graph 1). Five micrograms was the minimal quantity required to sensitize 1.0 ml. of a 0.5 per cent suspension of sheep erythrocytes so that agglutination with homologous serum prepared against the whole bacterial cells occurred at maximal titer. When quantities of 2.5 μg. and 1.25 μg. of the fraction were used, there was a decrease in titer of one and five serial serum dilutions, respectively.

The results obtained in the examination of the activity of specific serums are shown (table 2). Antiserum for strain UF, from whose cells sensitizing fraction HS was secured, agglutinated treated erythrocytes in a dilution of 1:20,000. With the exception of the serums for strains B-1 and B-4 of bovine origin, all other antiserums were capable of agglutinating the sensitized erythrocytes. The least degree of cross reaction was shown by antiserum for strain B-6, which agglutinated in a dilution of 1:320, while the maximum cross reaction was demonstrated by antiserum B-10, which reacted positively in a dilution of 1:20,000. The serums for two strains of ovine origin, Ill. and 53-D, agglutinated sensitized cells in dilutions of 1:5,120 and 1:1,280, respectively. No reactions, even in serum dilutions as low as 1:10, were shown by serums secured from 11 noninjected, apparently normal rabbits.

When the minimum dilution of antiserum that produced 4+ agglutination was incubated with free fraction HS for two hours, complete inhibition of hemagglutination was obtained with 1.5 to 12.0 μg. of the fraction in a volume of 0.4 ml. (table 3). Free fraction HS was capable of blocking agglutinating activity of serums produced against smooth and against rough variants. Hemagglutination by HS antiserums of strains UF and Ill. were prevented by free fraction HS of strain UF.

The effects of variation of incubating temperatures and variation of sensitizing dose of fraction HS on the hemagglutination titers of specific serums have been studied. The optimal results (table 4) were obtained when the tests were kept at room temperature throughout the entire incubation period. With slight variations, incubation at

TABLE 4—Joint Effects on Hemagglutination Titers of Varying Incubation Temperatures and Decreasing Amounts of Sensitizing Substance

Fraction HS * (μg./1.0 ml. of 0.5% cell suspensions)	Method of incubation	Dilution of antiserum of *V. fetus*, strain UF							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
20	18 hr. at room temperature.	4 †	4	4	4	4	1	1	1
	18 hr. at 4 C.	4	4	4	4	4	1	1	–
	2 hr. at 37 C., followed by 16 hr. at 4 C.	4	4	4	4	4	1	1	–
	2 hr. at 56 C., followed by 16 hr. at 4 C.	1	1	1	1	1	–	–	–
10	18 hr. at room temperature.	4	4	4	4	4	1	1	1
	18 hr. at 4 C.	4	4	4	4	4	1	1	1
	2 hr. at 37 C., followed by 16 hr. at 4 C.	4	4	4	4	3	1	1	–
	2 hr. at 56 C., followed by 16 hr. at 4 C.	1	1	1	1	1	–	–	–
5	18 hr. at room temperature.	4	4	4	4	4	1	1	1
	18 hr. at 4 C.	4	4	4	4	1	1	1	–
	2 hr. at 37 C., followed by 16 hr. at 4 C.	4	4	4	3	1	–	–	–
	2 hr. at 56 C., followed by 16 hr. at 4 C.	1	1	1	1	1	–	–	–
2.5	18 hr. at room temperature.	4	3	2	3	3	–	–	–
	18 hr. at 4 C.	4	4	3	–	–	–	–	–
	2 hr. at 37 C., followed by 16 hr. at 4 C.	1	1	1	1	–	–	–	–
	2 hr. at 56 C., followed by 16 hr. at 4 C.	–	–	–	–	–	–	–	–

* Fraction HS was obtained from *V. fetus* strain UF. † 4 = ++++; 3 = +++; 2 = ++; 1 = + (agglutination).

XUM

TABLE 5—Cross-Hemagglutination Reactions Among HS Antigens and Their Antiserums of Five Vibrio Fetus Strains

Strain origin	Biochemical activity: Catalase production	Biochemical activity: H_2S production	HS antigen	Antiserums against HS antigens: UF	4220	Ill.	6867	1134
Bovine	+	−	UF	10,000*	80	2,560	−	−
	−	+	4220	40	5,120	40	−	−
Ovine	+	−	Ill.	5,120	80	5,120	−	−
	−	+	6867	−	−	−	10,000	−
Human	+	−	1134	−	−	−	−	5,120

* Titer expressed as reciprocal of serum dilution. Fractions HS were prepared from smooth cells. Anti-HS serum was prepared by inoculating rabbits intravenously with 1 mg. of the fraction four times during the 21-day period.

4 C. and 37 C. revealed essentially identical results. Incubation at 56 C. resulted in weak reactions with lower serum dilutions. A sensitizing dose of 5 μg. of fraction per 1.0 ml. of 0.5 per cent erythrocyte suspension gave optimal serum titers. It was found that erythrocytes were sensitized more rapidly and to a somewhat greater degree at 37 C. than 24 C. The optimal adsorption was reached in about two hours at 37 C. The adsorption at 4 C. for six hours was only one fourth of optimum.

The HS fractions were secured from the cells of five different strains and used to sensitize sheep erythrocytes; the latter were tested with antiserums prepared against these fractions. The data on these reactions are compiled (table 5). A considerable degree of cross reaction was shown between HS fractions of strains UF and Ill., of bovine and ovine origin, respectively. Fraction HS of the catalase-negative, H_2S-positive strain 4220 of bovine origin demonstrated a slight degree of cross reaction with the fractions of strains UF and Ill. The fractions of catalase-negative, H_2S-positive strain 6867 of ovine origin and catalase-positive, H_2S-negative strain 1134 of human origin reacted with homologous serums only.

TABLE 6—Hemagglutination Reactions Among Three Vibrio Fetus Strains of Human Origin

Dilutions of serum in saline solution	Specific serums tested: 1134	1154	1307
1:20	++++*	++++	++++
1:40	++++	++++	+++
1:160	++++	++++	++
1:370	++++	++++	++
1:640	++++	+++	+
1:1,280	++++	++	.....
1:2,560	++++	+	.....
1:5,120	++	.....	.....
1:10,000	+	.....	.....

* Degree of agglutination reaction.
Erythrocytes were sensitized with HS fraction of strain 1134.

Erythrocytes sensitized with HS fraction of strain UF were agglutinated by serums representative of two serogroups as follows: Serum 543, serogroup V, of Price *et al.*,[17] agglutinated to a titer of 1:2,560, and serum R, serogroup I, of Kiggins,[9] agglutinated in a maximal dilution of 1:1,280. The other representative serums showed no serological reactivity with sensitized cells.

When the erythrocytes sensitized with HS fraction of strain 1134 of human origin were admixed with serums prepared against the whole cells of three strains of human origin, a considerable degree of cross reaction occurred (table 6). Homologous serum agglutinated to a titer of 1:10,000, while serums 1154 and 1307 agglutinated to dilutions of 1:2,560 and 1:640, respectively.

The results of hemagglutination tests on serums from 9 infected and 3 apparently normal bulls are shown (table 7). Four of six serums from animals which yielded catalase-positive vibrios reacted positively in dilutions of 1:160 to 1:640. Serums of 3 other animals, from which catalase-positive and catalase-negative or only catalase-negative vibrios were cultured, exhibited hemagglutination in dilutions of 1:80 to 1:320. Serums from bacteriologically negative bulls showed no agglutination of sensitized erythrocytes in dilutions of 1:20 or higher.

DISCUSSION

The observations recorded show that at least one heat-stable component, present in the HS fraction of *V. fetus*, can be adsorbed on intact sheep erythrocytes, rendering them specifically agglutinable by antibody directed against the adsorbed material. It

is recognized that antigens possessing this characteristic appear to have one common attribute—they are all polysaccharides.

Cross-hemagglutination reactions between erythrocytes sensitized with polysaccharides of strain UF and specific antiserums of strains of various origins and serogroups showed that this polysaccharide was possessed by many but not all *V. fetus* strains. This indicates that the HS substance may be a fraction of a type-specific O antigen common to a number of individual strains rather than a species-wide antigen. Landy [11] and Neter *et al.*[13] have shown that simultaneous or consecutive treatment of erythrocytes with two or more antigens resulted in hemagglutination by all antiserums as if the cells were treated with homologous polysaccharide only. Also, attempts to saturate the adsorbing erythrocyte surface by increasing many times the adsorbing dose failed. These observations suggested that different areas of the erythrocyte may be involved for each antigen. Therefore, hemagglutination should be conducted on greater numbers of strains to determine the antigenic characteristics of their polysaccharides. Perusal of results will indicate the type of polysaccharides which may simultaneously be adsorbed on cells to give a test with a broad serological activity.

The specific hemagglutination reaction has been inhibited by adding the soluble antigen to the serum before introducing the sensitized erythrocytes into the system. Under these circumstances the soluble antigen, if present in sufficient amounts, combines with its corresponding antibody and prevents it from agglutinating the sensitized erythrocytes. Extremely small amounts of free polysaccharides were capable of inhibiting specific hemagglutination reactions. Thus, this inhibition test, when utilized together with the hemagglutination test, permits the detection and quantitation of small quantities of the soluble antigen. It would be of interest to determine whether this technique permits the detection of specific antigen in cervicovaginal exudations of infected or recently recovered animals.

On the basis of the whole cell cross-agglutination reaction, King [10] found human strains of *V. fetus* type to be antigenically related. It also was shown that these strains shared their agglutinating antigens with certain *V. fetus* strains of animal origin.[10,23] In our study, three of the human strains of *V. fetus* type studied were found serologically related on the basis of their HS antigen. These organisms, however, did not share HS antigens with the animal strains studied.

It is generally understood that infection of a bull seldom results in the formation of serologically demonstrable serum agglutinins, thus presenting a difficult diagnostic problem. Plastridge *et al.*[16] suggested that this may be due to the low degree of pathogenicity of *V. fetus* for the genitourinary tracts of carrier bulls. The ability of polysaccharide-sensitized erythrocytes to react with serums from *V. fetus*-infected bulls

TABLE 7—Results of Hemagglutination Tests Conducted on Serum Samples of Vibrio Fetus-Positive and -Negative Bulls

Bull (No.)	Breed	Bacteriological finding	Serum dilutions 1:20	1:40	1:80	1:160	1:320	1:640
H233	Holstein-Friesian	C+ Vibrio	++++*	++++	+++	++	−	−
J127	Jersey	C+ Vibrio	++++	++++	++++	++++	+++	+
809	Aberdeen Angus	C+ Vibrio	++++	++++	++++	++++	++	+
H211	Holstein-Friesian	C+ Vibrio	−	−	−	−	−	−
J141	Jersey	C+ Vibrio	−	−	−	−	−	−
G323	Guernsey	C+ Vibrio	++++	+++	++++	+	−	−
G330	Guernsey	C+ and C− Vibrio	++++	++++	++++	+++	+++	−
G335	Guernsey	C− Vibrio	++++	++++	+++	++	+	−
J140	Jersey	C− Vibrio	++++	++++	+++	−	−	−
H242	Holstein-Friesian	No Vibrio	−	−	−	−	−	−
H244	Holstein-Friesian	No Vibrio	−	−	−	−	−	−
J151	Jersey	No Vibrio	−	−	−	−	−	−

* Degree of agglutination reaction.

Erythrocytes were sensitized with HS fraction of strain UF. C+ = catalase-positive; C− = catalase-negative.

XUM

might be due to the capacity of this system to detect lower concentrations of circulating antibodies.

It also is possible that the extracted HS antigen contains, in addition to the principal antigen, certain secondary or minor antigenic components which, when adsorbed on the erythrocyte surface, are rendered reactive. The greater sensitivity of the hemagglutination test may be due further to the ability of treated erythrocytes to detect so-called "univalent" or incomplete antibody, as shown to be present in various immune serums.[7,14] Such antibody has the power of combining with antigen but does not form aggregates. Borduas and Grabar [3] referred to this antibody as "nonflocculating" and they showed the ability of hemagglutination methods to detect it. Failures of the tests to detect the antibodies in serums of 2 infected bulls might be due to antigenic difference of HS fraction of strains responsible for infection of these 2 animals.

Recent studies by Frank *et al.*[6] showed that bulls infected with catalase-negative strains of Vibrio are capable of infecting the female during a breeding season. Recent experiments revealed that serum antibody of bulls infected with catalase-negative Vibrio agglutinated erythrocytes sensitized with HS fraction of catalase-positive organisms. This is in agreement with an earlier observation that *V. fetus* shares certain heat-stable antigens with catalase-negative Vibrio.[21] Under these circumstances, the hemagglutination test could be used on greater numbers of animals as a screening diagnostic procedure, and bacteriological methods might then be used for more detailed examinations of individual, serologically positive animals.

To evaluate futher the possible usefulness of this serological technique in the field, it will be necessary to experimentally infect a number of cattle of both sexes to compare their pre- and postinoculation titers and to correlate the presence of the antibody with the bacteriological findings. The apparent advantages of the purified antigen described over whole Vibrio cells for serological use are its uniformity, great stability, and the ease and precision with which it may be standardized. In addition, the method is convenient, the reactions are clear, and the requirements for serological apparatus and techniques are simple.

SUMMARY

A hemagglutination test has been developed for detection of antibody against *Vibrio fetus*. Sheep erythrocytes sensitized with a polysaccharide fraction of *V. fetus* have been agglutinated by specific serums produced in rabbits and by serums of naturally infected bulls. The fraction apparently represents a type-specific O antigen common to a number of individual strains, rather than a species-wide antigen. The specificity of the hemagglutination reaction was shown by its inhibition by the free polysaccharide.

Some advantages, characteristics, and principles concerning the hemagglutination test are discussed.

References

[1] Alexander, M. M., Wright, G. G., and Baldwin, G. G.: Observations on the Agglutination of Polysaccharide Treated Erythrocytes by Tularemia Antisera. J. Exptl. Med., *91*, (1950): 561–566.

[2] Bond, T. M.: Characteristics of Colonial Forms of *Vibrio Fetus*. Am. J. Vet. Res., *18*, (April, 1957): 449–455.

[3] Borduas, A. G., and Grabar, P.: L'Hemagglutination passive dans la recherche des anticorps antiproteiques. Ann. Inst. Pasteur, *84*, (1953): 903–923.

[4] Boyden, S. V.: Adsorption by Erythrocytes of Antigens of *Pfeiferella Mallei* and *Pfeiferella Whitmori*. Proc. Soc. Exptl. Biol. & Med., *73*, (1950): 288–291.

[5] Bukantz, S. C., Rein, C. R., and Kent, D. R.: Studies in Complement-Fixation. II. Preservation of Sheep's Blood in Citrate Dextrose Mixtures (Modified Alsever's Solution) for Use in the Complement-Fixation Reaction. J. Lab. & Clin. Med., *31*, (1946): 394–399.

[6] Frank, A. H., Bryner, J. H., and Caruthers, B.: A Comparison of the Effect of Catalase-Positive and Catalase-Negative Vibrio on the Fertility of Cattle. Am. J. Vet. Res., *19*, (Jan., 1958): 108–111.

[7] Heidelberger, M., Treffers, H. P., and Mayer, M.: A Quantitative Theory of the Precipitin Reactions. VII. The Egg Albumin Antibody Reaction in Antisera from the Rabbit and Horse. J. Exptl. Med., *71*, (1940): 271–282.

[8] Keogh, E. V., North E. A., and Warburton, U. F.: Hemagglutinins of Haemophillus Group. Nature, *160*, (1947): 63.

[9] Kiggins, E. M.: Antigenic Characteristics of *Vibrio Fetus*, and Their Relation to Other Vibrios, *Brucella Abortus*, *Pasteurella Tularensis* and *Salmonella Pullorum*. Ph.D. thesis, University of Connecticut, Storrs, 1954.

[10] King, E. O.: Human Infections with *Vibrio Fetus* and a Closely Related Vibrio. J. Infect. Dis., *101*, (1957): 119–128.

[11] Landy, M.: On Hemagglutination Procedures Utilizing Isolated Polysaccharides and Protein Antigens. Am. J. Pub. Health, *44*, (1954): 1059–1064.

[12] Lank, R. B.; Louisiana State University, Baton Rouge: Personal communication, 1958.

[13] Neter, E., Bertram, L. F., Zah, D. A., Merdoch, M. R., and Argerman, C. E.: Studies on Hemagglutination and Hemolysis by *Escherichia Coli* Antisera. J. Exptl. Med., *96*, (1952): 1–15.

[14] Pappenheimer, A. M.: Anti-Egg Albumin Antibody in the Horse. J. Exptl. Med., *71*, (1940): 263–269.

[15] Plastridge, W. N.; University of Connecticut, Storrs: Personal communication, 1958.

[16] Plastridge, W. N., Walker, E. C., Williams, L. F., Stula, E. F., and Kiggins, E. M.: Isolation of *Vibrio Fetus* from Bulls. Am. J. Vet. Res., *18*, (July, 1957): 575–583.

XUM

[17] Price, K. E., Poelma, L. J., and Faber, J. E., Jr.: Serological and Physiological Relationships Between Strains of *Vibrio Fetus*. Am. J. Vet. Res., *16*, (Jan., 1955): 164–169.

[18] Ristic, M., and Brandly, C. A.: Characterization of *Vibrio Fetus* Antigens. I. Chemical Properties and Serological Activities of a Soluble Antigen. Am. J. Vet. Res., *19*, (Oct., 1958): In press.

[19] Ristic, M., Herzberg, M., and Sanders, D. A.: Colonial and Antigenic Variations of *Vibrio Fetus*. Am. J. Vet. Res., *17*, (Oct., 1956): 803–809.

[20] Ristic, M., White, F. H., and Doty, R. B.: Morphological and Serological Characteristics of Fresh Isolates of *Vibrio Fetus* and Other Vibrios Inhabiting the Bovine and Ovine Genital Tract. Am. J. Vet. Res., *19*, (Jan., 1958): 99–107.

[21] Ristic, M., White, F. H., Doty, R. B., Herzberg, M., and Sanders, D. A.: The Characteristics of Agglutinating Antigens of *Vibrio Fetus* Variants. I. Effect of Heat and Formalin on Serological Activity. Am. J. Vet. Res., *18*, (Oct., 1957): 764–770.

[22] Ristic, M., Sanders, D. A., and Tyler, M. E.: *Vibrio Fetus* Infection in Cattle. I. A Comparative Study of the Agglutinin Response in Cervicovaginal Mucus and Blood Serum to Homologous Antigens Following Abortion. Am. J. Vet. Res., *16*, (April, 1955): 246–250.

[23] Ristic, M., Sanders, D. A., and Young, F.: Experimental *Vibrio Fetus* Infection in Male Hamsters. Am. J. Vet. Res., *16*, (Jan., 1955): 189–192.

Studies on an Immunogenic Agent Against *Brucella Abortus* I. Titrations of a New Soluble Immunogenic Agent Against Experimental Infections in Guinea Pigs

WARREN G. HOAG, D.V.M., M.P.H., and ROBERT C. ALLEN, M.S.

Blacksburg, Virginia

THE WORK of Hoag and Bell[7] demonstrated a method of inducing immunity against *Leptospira pomona* infections without producing significant levels of agglutinins in the serum of vaccinated cattle. With regard to preventive methods in diseases in which diagnosis depends on the detection of circulating antibody, the possibility that immunogenic agents may produce diagnostically confusing antibody reactions in vaccinated animals must be considered. This is particularly important in diseases in which there are a high percentage of subclinically infected animals which develop into carriers.

Brucellosis control in this country is dependent upon two basic procedures: the elimination of infected cattle and the vaccination of young herd replacements. Considerable reliance is placed upon the presence of specific serum agglutinins in blood and milk[3] as a means of detecting infected animals to be eliminated. This has been complicated by the fact that agglutinins are not only produced by the naturally occurring disease, but also by vaccines.[10,11] For this reason, it is recommended that cattle be vaccinated during calfhood (4 to 8 months of age) so that agglutinin titers probably will have subsided by the time the animal reaches adulthood.[2,5] In this way, it is possible to develop serologically negative herds. It can be assumed that significant agglutinins developing after this time are a result of natural infections.

This procedure precludes revaccination for booster purposes because of the danger of producing agglutinins which will necessitate elimination of the animal from the herd and the risk of not being able to differentiate vaccinated from naturally infected animals. In spite of recent improvements in diagnostic procedures to help eliminate a portion of this problem,[1,4,8] the major dangers still exist. It was with these problems in mind that this work was undertaken.

MATERIALS AND METHODS

Experiment 1.—Brucella abortus strain 2308* was grown in 300 ml. of Stuart's liquid leptospiral medium for 48 hours at 37 C., under partial CO_2 tension.[7] The culture (1×10^8 viable organisms/ml.) then was heated to 100 C., at which time 0.1 M acetic acid was added in an amount equal to 5 per cent of the total culture volume. Heating continued for another five minutes, until the precipitation of proteinaceous material was complete. After cooling under running tap water, the precipitate was removed by centrifugation (1,500 r.p.m.), suspended in distilled water, and enough 0.1 M NaOH added to neutralize and dissolve the material. The final solution corresponded to one fifth of the original culture volume. Merthiolate (Thimerosal†), to a final concentration of 1:10,000, was added as a preservative, and an adjuvant of 6 per cent lanolin in mineral oil was mixed with the product. This was designated "lot 1" vaccine.

Sixty-four male guinea pigs,** weighing approximately 250 Gm. each, were allotted at random to 16 groups of 4 animals each for a "box-type" titration of varying amounts of vaccine against varying dilutions of challenge material (table 1). Those given vaccines were inoculated subcutaneously in the cervical region.

The challenge material consisted of *Br. abortus* strain 2308 grown on Albimi's Brucella agar under partial CO_2 tension. The 48-hour growth was washed from the agar surface with sterile physiological saline solution and rewashed once by centrifugation. The organisms then were standardized in saline solution to a concentration equal to No. 1 of the MacFarland nephelometer series. Dilutions of this suspension were made in saline solution at 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}. On the twelfth day after vaccination, 1 ml. of a dilution was given intraperitoneally as challenge material.

From the Animal Pathology Section, Virginia Agricultural Experiment Station, Blacksburg. Dr. Hoag is now at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

Portions of the work were done in partial fulfillment of the requirements for the Ph.D. degree (Allen).

The authors acknowledge the technical assistance of Jean Kerstetter and the advice of Dr. James R. Rooney.

* Obtained from the Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

† Thimerosal is produced by Eli Lilly and Co., Indianapolis, Ind.

** Obtained from the Albino Farms, Red Bank, N.J.

TABLE 1—Titrations of Varying Amounts of Vaccine Against Varying Dilutions of Challenge Material (Experiment 1)

Challenge dilutions	Amounts of vaccine			
	None	0.25 ml.	0.50 ml.	1.00 ml.
10^{-1}	A*	E	I	M
10^{-3}	B	F	J	N
10^{-5}	C	G	K	O
10^{-7}	D	H	L	P

* 4 guinea pigs per letter.

The protocol of titration of infective organisms against the varying doses of vaccine is shown (table 1).

Blood samples were taken for serological examination from all guinea pigs, by heart puncture, two days before they were challenged by living organisms (10 days postvaccination) and again 28 days after challenge (2.0 ml. of blood was removed from each). Tube-agglutination techniques and materials used were according to standard methods recommended by the U. S. Department of Agriculture.

Animals were observed for 28 days after being given the challenge material, and any deaths or abnormalities were recorded. All were killed at the end of this period, and any gross pathological changes were recorded. At the same time, ground spleen and liver from each guinea pig were inoculated into Albimi's semisolid agar and incubated at 37 C. under partial CO_2 tension. Subcultures from those tubes showing any bacterial growth then were made on basic fuchsin and thionin plates incubated under CO_2 tension. Final identification of Brucella was made serologically, using adsorbed homologous rabbit antiserum to identify the isolates positively as strain 2308.

Experiment 2.—Brucella abortus strain 2308 was inoculated into Stuart's medium as in experiment 1, except that the material was grown for 13 days at 37 C. instead of for 48 hours. The culture material (1×10^7 viable organisms/ml.) then was acid-heat-treated in the same way as in experiment 1. Reconstitution, preservation, and addition of adjuvant also followed the same procedures. This lot of vaccine was designated "lot 2."

Five guinea pigs per group, instead of 4, were used for this experiment. Other procedures, including source and selection of animals, bacteriological, serological, and vaccination techniques, were the same as for experiment 1. The only other difference between procedures used was that challenge was made 21 instead of 12 days after vaccination. The diagram of titration in experiment 2 is shown (table 2).

TABLE 2—Titrations of Varying Amounts of Vaccine Against Varying Dilutions of Challenge Material (Experiment 2)

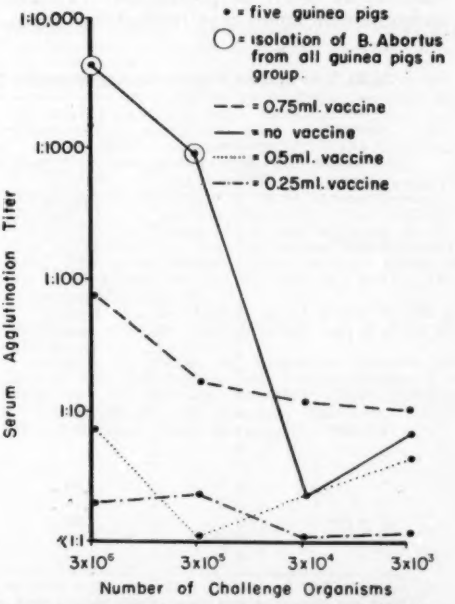
Challenge dilutions	Amounts of vaccine			
	None	0.25 ml.	0.50 ml.	0.75 ml.
10^{-2}	A*	E	I	M
10^{-3}	B	F	J	N
10^{-4}	C	G	K	O
10^{-5}	D	H	L	P

* 5 guinea pigs per letter.

RESULTS

Experiment 1.—The titration of the challenge material in the groups of guinea pigs given no immunogenic agent resulted in an $i.d._{50}$ (infective dose for 50% of animals) of approximately 110×10^3 organisms per animal.[9] Serums taken before and ten days after vaccination were negative to the lowest dilution (1:20) of the tube-agglutination test system. Titration of the challenge material in the groups of guinea pigs given 0.25 ml., 0.50 ml., and 1.00 ml. of immunogenic agent gave $i.d._{50}$'s of 200×10^3, 300×10^3, and 2×10^3, respectively. This indicated that no protection resulted. Titrations were based on isolation of *Br. abortus* strain 2308 from tissues of animals at necropsy, and not from positive serological tests.

Experiment 2.—The results of experiment 2 with lot 2 vaccine, are shown (table 3). On the basis of isolations of the organisms from necropsy material, it will be seen that, in guinea pigs given no vaccine, the



Graph 1—Geometric mean serum-agglutination titers of guinea pigs in experiment 2. Serums were collected 28 days after challenge.

i.d.$_{50}$ of challenge was approximately 165 $\times 10^3$ organisms. In the groups of animals given the smallest dose of vaccine (0.25 ml.), the i.d.$_{50}$ end point was greater than 3,000 $\times 10^3$ organisms. The protective index (difference between i.d.$_{50}$'s for control and vaccinated animals) was greater than 2.5 logs. This was true for all dosages of the immunogenic agent.

All serums from guinea pigs in the groups given the smallest dose of immunogenic material had tube-agglutination titers of less than 1:20 when tested 19 days after vaccination (2 days before challenge), with the exception of 1 animal whose serum had a titer of 1:40 at this time. The larger doses (0.5 ml. and 0.75 ml.) of the agent produced a greater frequency of positive titers equal to or greater than 1:20 (4/20 and 15/20, respectively) and higher individual titers (geometric means of 1:2.3 and 1:16, respectively).

Geometric mean titers for each group of guinea pig serums (individual tests, not pooled) are shown (graph 1).

DISCUSSION

The results obtained in the first experiment indicated that either or both of two conclusions could be postulated: (1) The immunogenic agent was ineffective in producing immunity against the homologous agent, or (2) an immunogenic agent of this nature could be effective against the homologous organism, provided certain conditions of the experiment were modified.

The second postulate was considered along the following lines: It had been shown in previous work that the effectiveness of the immunogenic agent produced by this method was determined by the amount of bacterial metabolic by-product present in the culture liquor, rather than solely by the total members of intact organisms at the time of preparation. It also was possible that the time between vaccination and challenge in experiment 1 was too short. *Brucella abortus* strain 2308 is supposedly more virulent for the guinea pig than is reported here. Apparently there was some loss of bacterial virulence before its initial use in these experiments, since the i.d.$_{50}$'s of organisms remained appreciably the same for both experiments, which were several months apart. It is possible that host factors (such as the strain of guinea pig and nutritional status) could be part of the explanation for the degree of virulence reported here.

The degree of immunity produced by the immunogenic agent in experiment 2 leaves no doubt that an effective means of

TABLE 3—Guinea Pig Serum-Agglutination Titers Before and After Challenge (Experiment 2)

No. organisms in challenge dose	Guinea pig (No.)	Amounts of vaccine							
		None		0.25 ml.		0.50 ml.		0.75 ml.	
		C*	C + 28†	C	C + 28	C	C + 28	C	C + 28
3×10^6	1	—	1,280**	—	—	—	20	160	160
	2	—	2,560**	—	—	—	—	20	40
	3	—	2,560**	—	—	—	40	—	20
	4	—	1,280**	—	20	80	—	80	160
	5	—	640**	—	—	—	20	20	160
3×10^5	1	—	320**	—	—	—	—	20	20
	2	—	1,280**	—	—	—	—	40	40
	3	—	640**	—	—	40	—	20	—
	4	—	Died‡	—	—	—	—	40	40
	5	—	2,560**	—	40	—	—	—	40
3×10^4	1	—	—	40	—	—	—	40	20
	2	—	40	—	—	—	—	—	—
	3	—	—	—	—	—	20	40	40
	4	—	—	—	—	80	20	160	—
	5	—	—	—	—	—	—	80	160
3×10^3	1	—	20	—	—	80	80	40	40
	2	—	—	—	—	—	—	20	40
	3	—	20	—	—	—	—	—	—
	4	—	20	—	—	—	20	40	40
	5	—	—	—	—	—	—	—	—

* C = two days before challenge; † C + 28 = 28 days after challenge; ** *Brucella abortus* was isolated from the tissues at this time; ‡ this animal died immediately before blood samples were taken and *Br. abortus* was isolated from the tissues at this time.

Titers are expressed as reciprocal of highest dilution showing 2+ agglutination; — = no agglutination at lowest serum dilution (1:20).

immunizing guinea pigs can be produced by this method of extraction. It is probable that the determining factor between the success of lot 2 over lot 1 vaccine lies in the age of the culture used. Supporting evidence for this is shown (table 3) by the serological results of experiment 2, in which large doses of the vaccine did produce agglutinins, while the same large doses of vaccine (lot 1, experiment 1) made from cultures only 48 hours old produced no agglutinins. It is not proposed that the presence of agglutinins in blood serum is an indication of immunity, but rather that it is an indication of vaccine antigenicity. The results of experiment 2 indicate that immunogenically effective doses of vaccine can be administered with no development of appreciable levels of serum agglutinins. The geometric mean titers are also an indication of this effect (*see* lines for various dosages of vaccine, graph 1), with no appreciable boost from challenge organism inoculation (table 3, experiment 2).

The method of preparation of the immunogenic agent utilizes the metabolic by-products of bacterial growth, in addition to the bacterial cells. The technique involves the extraction of proteinaceous material derived from the bacterial cells, metabolic by-products, and the rabbit serum contained in the growth medium. It is possible to postulate that other fractions, such as carbohydrates, adhere to the surface of protein masses obtained in this way.[6] The method serves to extract immunogenic material and to destroy or to leave behind agglutinogenic substances. This is important to the preparation of any vaccine, in that such immunogenic agents may be used in most host situations (such as age and previous immune status) without serum-agglutinin response. This is particularly important for bovine brucellosis.

SUMMARY

A soluble-type vaccine was prepared by the acid-heat extraction of *Brucella abortus* strain 2308 and its metabolic by-products in Stuart's medium. A comparison was made, in guinea pigs, of 2-day-old and 13-day-old cultures for the preparation of the immunogenic agent. Although the number of viable organisms present in the culture liquid was approximately the same for both lots, there was a significant difference in immunogenic activity. The agent made from the 2-day-old culture produced no significant protection against various dosage levels of living *Br. abortus* strain 2308 organisms. The agent prepared from the 13-day-old culture produced not only significant protection against homologous strain challenge but, at effective dosage levels, produced insignificant serum-agglutination titers. Protection was determined by the failure to isolate Brucella organisms in tissues taken at necropsy.

References

[1] Barner, R. D., Oberg, F. H., and Atkinson, F. W.: Anamnestic Reaction as an Aid in the Differentiation of Vaccinal from Infection Titers of Brucellosis *(Abortus)* in Cows. Vet. Med., *47*, (1952): 327.

[2] Birch, R. R., Gilman, H. E., and Stone, W. S.: The Immunity Created by Vaccination of Calves with *Brucella Abortus* Strain 19. Cornell Vet., *35*, (1945): 110.

[3] Blake, G. E., and Manthei, C. A.: The Efficiency of the Blood, Whey, and Whole Milk Tests in Ascertaining the Brucellosis Status of Cattle. Proc. Book, AVMA (1952): 98.

[4] Dick, J. R., Venzke, W. G., and York, C. A.: A Method for Differentiating Between Vaccinal Titers and Infection Titers of *Brucella Abortus* in Cattle. J.A.V.M.A., *111*, (Oct., 1947): 255–258.

[5] Haring, C. W., and Traum, J.: The Effect of *Brucella Abortus* Strain 19 on Cattle of Various Ages and Its Bearing on Adult Cattle Vaccination. Proc. U.S. Livestock San. A. (1943): 42–46.

[6] Hoag, W. G., and Allen, R. C., Virginia Agricultural Experiment Station: Unpublished data, 1955.

[7] Hoag, W. G., and Bell, W. B.: An Immunogenic Agent for the Protection of Cattle Against *Leptospira Pomona*. Am. J. Vet. Res., *16*, (July, 1955): 381–385.

[8] King, N. B., Venzke, W. G., and Edgington, B. H.: The Application of a Suggested Method for Differentiating Vaccinal and Infection Titers in Cattle Known to Be Infected with *Brucella Abortus*. J.A.V.M.A., *123*, (Aug., 1953): 121–124.

[9] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493.

[10] Sieiro, F.: Studies on Comparative Brucella Immunity with Agglutinogenic and Nonagglutinogenic Vaccines. Am. J. Vet. Res., *15*, (July, 1954): 417–424.

[11] Spink, W. W.: The Nature of Brucellosis. University of Minnesota Press, Minneapolis, 1956.

XUM

Field Evaluation of *Clostridium Botulinum* Type C Toxoids in Mink

GEORGE S. APPLETON, M.S., and PHILIP G. WHITE, Ph.D.

Madison, Wisconsin

IN A PREVIOUS REPORT,[2] laboratory variables affecting toxin production and evaluation of toxoids prepared from *Clostridium botulinum* type C cultures were initiated prior to beginning field trials with type C toxoids in mink.

In recent years, several workers have investigated the efficacy of type C toxoids in protecting mink against type C intoxication. Kull and Moberg,[3] Larsen *et al.*,[4] and Appel *et al.*[1] demonstrated the practicality of immunizing mink with type C adsorbate toxoids.

The purpose of this work was to determine the value of several experimental type C toxoid preparations in protecting mink under field conditions against artificially induced type C intoxication. For correlative purposes, the relative immunity responses of mice and mink to type C toxoids were studied.

MATERIALS AND METHODS

Toxoids.—The preparation of the toxoids has been previously described.[2] Toxoids were prepared from 5- to 10-day-old cultures grown in intussuscepted membranes* by adding 0.5 per cent commercial grade formalin and reincubating at 37 C. for eight to 12 days. The toxoids were adsorbed in a manner similar to that described by Rice,[7] and were adjusted to contain varying amounts of antigen. A total of six toxoid preparations was used. A seventh preparation, *Clostridium perfringens* type D bacterin, which had approximately the same physical characteristics as the experimental toxoid preparations, was used as a placebo.

Coding of Toxoids.—Each of the toxoids was coded to preclude any bias in the handling or interpretation of results by the cooperating veterinarians* who vaccinated and challenged the immunity of all mink.

Procedure.—For the mink trials, one complete set of coded toxoid preparations, consisting of six toxoid lots and one placebo, was used for each replication. A total of ten replications was conducted in three states by three operators. Each replication required 70 mink (10 mink for each of the toxoids and 10 for the placebo). Approximately 700 mink were included in the study.

Each mink was injected subcutaneously in one of the axillary spaces with 1.0 ml. of the respective toxoid or placebo and records were made as to the age and sex of the animal. All animals were injected during the months of July, August, and September, 1956. After vaccination, several animals in each group were examined for postvaccination reactions.

For correlative data in mice, 280 healthy white males, weighing 20 to 30 Gm., were used. Two replications of 140 mice each (20 mice for each toxoid lot and 20 for the placebo) were made. Each mouse was given 0.5 ml. of toxoid or placebo intraperitoneally. The immunity of all mice was challenged 14 days postvaccination with standardized lyophilized type C toxin.

Mink Challenge.—At pelting time, all mink were held overnight without feed and then were challenged with a standardized lyophilized whole culture of *Cl. botulinum* type C toxin. A small patty of regular mink feed was inoculated with 0.5 ml. of the toxin and mixed into the feed. The animals were fed the inoculated feed immediately and all signs of illness and deaths were then recorded for 96 hours. The challenge dose was calculated to be approximately 20 mink $l.d._{50}$, but actually represented greater than 100 mink $l.d._{50}$ (table 1). The $l.d._{50}$ end points were calculated according to the method described by Reed and Muench.[6]

Animals.—Of the mink vaccinated with the various toxoid preparations, 391 were males (390 kits and 1 adult) and 299 were females (198 kits and 101 adults).

RESULTS

In cooperation with one of the veterinarians (Hartsough), the standardized lyophilized toxin was titrated in mink at the time of challenge. The toxin was restored in the accompanying diluent (0.2% gelatin in phosphate buffer at pH 6.5[5]) and tenfold and 100-fold dilutions were made in physiological saline solution. Mink feed patties were prepared as before, and 0.5 ml. of the respective toxin dilutions was injected and thoroughly mixed into each of 13 patties for each dilution. These were immediately fed to female mink, 6 months old, which had been held for 24 hours without feed. The results of the titration of the challenge toxin are given (table 1).

From the Research Division, American Scientific Laboratories, Inc., Madison, Wis.

* The authors thank Drs. G. R. Hartsough, Pittsville, Wis., Stewart Burkhardt, Lakewood (Denver), Colo., and Austin E. Larsen, Salt Lake City, Utah, for their cooperation in conducting the field trials.

TABLE 1—Titration of Standardized Challenge Toxin in Mink (Per Os)

Toxin dose	0.5 ml.	0.5×10^{-1} ml.	0.5×10^{-2} ml.
Death ratio	13/13*	13/13	12/13

* Numerator = No. dead; denominator = No. challenged.

One of the veterinarians (Larsen) reported that the challenge toxin at a dilution of 1:100 killed nonvaccinated mink (number not specified) in 48 hours. The results of the challenge of the immunity of all mink vaccinated with the *Cl. botulinum* type C toxoids are shown (table 2).

In addition to the evaluations reported (table 2), one of the veterinarians (Larsen) vaccinated 1,000 mink (age and sex not specified) with 1.0 ml. of a toxoid preparation similar to FDA in table 2. The toxoid used was supplied by this laboratory. At pelting time, the immunity of 50 of the 1,000 mink was challenged with 0.5 ml. of the undiluted standardized toxin injected into the feed; 49 survived. There were no adverse reactions to the vaccination in any of the 1,000 mink.

The cumulative results of the immunity challenge of the mice vaccinated with the various toxoid lots are given (table 3). The $l.d._{50}$ of the toxin used in challenging the immunity of the mice was obtained from the placebo end point.

The correlation between the immune responses of mice and mink when artificially exposed to type C intoxication is indicated (graph 1).

DISCUSSION

It has been reported [1, 4] that 1,000 to 10,000 mouse intraperitoneal $l.d._{50}$ are approximately equivalent to 1 mink oral $l.d._{50}$. In establishing the challenge dose for the field trials reported here, it was desired to challenge the immunity of the mink with approximately 200,000 mouse $l.d._{50}$, or about 20 mink $l.d._{50}$. From the results shown in table 1, it is evident that the challanged mink were given more than 100 $l.d._{50}$.

In general, the immune response in mink against the massive challenge dose with five of the preparations was excellent. The one toxoid lot that gave a "poor" response (lot FDB) still protected 79 per cent of the mink vaccinated.

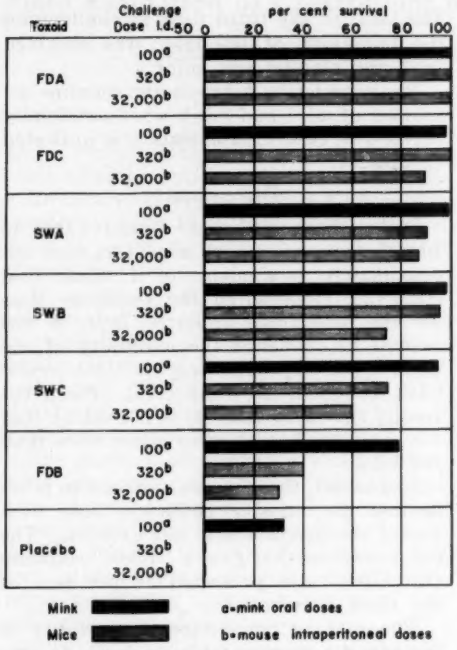
The varying percentage of mortality in the placebo group (table 2, ranch 4) was not interpreted as indicating any cross

TABLE 2—Summary of Field Trial Challenge Results, All Replications

Ranch	Sex	Experimental toxoid groups							
		FDA	FDB	FDC	SWA	SWB	SWC	Placebo	Controls
1	M	.....	.....	.....	.....	.....	.....	.....	.....
	F	0/10*	0/10	0/10	0/10	0/10	2/10	7/10	50/50
2	M	0/5	3/10	0/5	0/5	0/5	0/5	5/5	.....
	F	0/5	.....	0/5	0/5	0/5	0/5	1/1	.....
3	M	0/5	3/5	0/5	0/5	0/5	0/5	2/5	.....
	F	0/5	2/5	0/5	0/5	0/5	0/5	2/5	.....
4	M	0/10	3/10	0/10	0/10	0/10	0/10	1/10	.....
	F	.....	.....	.....	.....	.....	.....	.....	.....
5	M	0/10	0/10	2/10	0/10	1/10	2/9	9/10	.....
	F	.....	.....	.....	.....	.....	.....	.....	.....
6	M	0/5	0/5	0/5	0/5	0/5	0/5	4/5	.....
	F	0/5	0/5	0/5	0/5	0/5	0/5	0/5	.....
7	M	.....	0/10	0/10	.....	.....	0/10	9/10	.....
	F	0/10	.....	.....	0/10	0/10	.....	.....	.....
8	M	.....	.....	0/10	0/8	0/10	0/4	.....	.....
	F	0/9	3/10	.....	0/2	.....	0/5	9/9	.....
9	M	0/10	1/10	0/10	0/10	1/10	2/10	7/10	.....
	F	.....	.....	.....	.....	.....	.....	.....	.....
10	M	.....	.....	.....	.....	.....	.....	.....	.....
	F	0/10	6/10	0/10	1/10	0/8	0/10	9/10	11/11
MALES									
Dead		0	10	2	0	2	4	37	.....
Challenged		45	60	65	53	55	58	55	.....
Percentage		0.0	16.7	3.1	0.0	3.6	6.9	67.0	.....
FEMALES									
Dead		0	11	0	1	0	2	28	61
Challenged		54	40	35	47	43	40	40	61
Percentage		0.0	27.5	0.0	2.1	0.0	5.0	70.0	100.0
ALL MINK									
Dead		0	21	2	1	2	6	65	61
Challenged		99	100	100	100	98	98	95	61
Percentage		0.0	21.0	2.0	1.0	2.1	6.1	68.5	100.0

*Numerator = No. dead; denominator = No. challenged.

XUM



Graph 1—Correlation of immune responses in mice and mink to Clostridium botulinum type C toxoids.

immunogenicity between the antigens of *Cl. botulinum* type C and *Cl. perfringens* type D. Judging from observations made during the immunity challenge on ranches 9 and 10, it was believed that the variability was the result of faulty technique in administering the challenge toxin. The toxin, when injected into the wet feed patties, was not easily adsorbed and "leaked" out of the feed. Furthermore, in several instances part of the feed was lost through the wire floor of the mink pen before being consumed. It is possible that a large portion of the toxin could have been lost in this manner.

In later titrations (control groups, table 2), the toxin was thoroughly mixed in a small amount of feed and fed to mink previously starved for 24 hours. These results were more consistent.

It was also established (graph 1 and table 3) that there is no cross immunogenicity between the antigens of *Cl. botulinum* type C and *Cl. perfringens* type D.

The results obtained in these trials indicate that male mink may be slightly more resistant to type C intoxication than are females. The difference, however, is not considered significant. The individual records of the results on each ranch did not indicate that there was any difference in susceptibility to type C intoxication based on the age of the mink, *viz.*, at a defined challenge dose, adult mink appear to be as susceptible as kits.

The interval from vaccination to challenge for all ranches varied from 62 to 139 days. The 62-day interval is represented by ranch 8 and the 139-day interval by ranch 7. The average interval for all ranches was 94 days. From a practical standpoint, this means that kits vaccinated when separated from the dams (usually in July) should remain resistant to type C intoxication at least until pelting time (usually in December). It is probable that the duration of immunity after vaccination with one 1.0-ml. dose of type C toxoid is at least two years.[3]

Data in table 3 and graph 1 indicate that mice are more sensitive as test animals for evaluating type C toxoids than mink. The

TABLE 3—Evaluation of Clostridium Botulinum Type C Toxoids in Mice—Cumulative Challenge Results from Two Replicates in Mice

Calculated l.d.50*	Toxoid lot						
	FDA	FDB	FDC	SWA	SWB	SWC	Placebo†
32,000.0	0/39**	28/40	4/36	5/35	10/39	15/37	.....
	0	70	11	14	26	41	
3,200.0	0/30	19/30	1/28	3/28	5/30	10/30	.....
	0	63	3.6	11	17	33	
320.0	0/20	12/20	0/20	2/20	1/20	5/20	30/30
	0	60	0	10	5	25	100
32.0	0/10	7/10	0/10	0/10	0/10	2/10	20/20
	0	70	0	0	0	20	100
3.2	.....	.....	.....	.....	.....	.....	10/10
							100
<1.0	.....	.....	.....	.....	.....	.....	0/10
							0

* The l.d.50 was calculated from the placebo end point; † placebo = *Clostridium perfringens* type D bacterin; ** numerator = No. dead/No. challenged (cumulative), denominator = percentage of deaths.

correlation between the immune responses of mice and mink was good. Toxoid FDB was rejected as being a poor antigen in mink (79% protection) as well as in mice (30% protection against a challenge dose of 32,000 l.d.$_{50}$). The varying ability to stimulate immunity in mink with the other toxoid preparations was paralleled in mice. It is conceivable that a toxoid that was satisfactory in mink would be rejected when tested in mice.

None of the toxoid lots caused visible local or systemic reactions, and examination of the pelt at pelting time demonstrated that there was no damage. With proper vaccination techniques, no residual nodule was evident that might cause the pelt to rip when fleshed.

SUMMARY

Six *Clostridium botulinum* type C toxoids, prepared from parent toxin produced in two different mediums and containing varying amounts of antigenic material, were evaluated in mink under field conditions. All except one of the toxoid lots stimulated good immunity responses in mink exposed to more than 100 mink l.d.$_{50}$ of *Cl. botulinum* type C toxin administered *per os*.

The vaccinated mink were resistant to challenge for at least 139 days. The challenge dose killed 68 per cent of 95 mink vaccinated with a placebo and 100 per cent of 61 nonvaccinated control mink. Observations were made as to why greater mortality was not obtained in the placebo group.

There was little indication of variance in susceptibility to type C intoxication among male kits, female kits, and female adults.

A definite correlation between the immune responses in mice and mink to *Cl. botulinum* type C toxoids was established.

References

[1] Appel, R. N., Hartsough, G. R., McCoy, E. F., and Brandly, C. A.: Active and Passive Protection of Mink Against Type C Botulism with Type C Toxoid and Polyvalent Antitoxin. J.A.V.M.A., *128*, (June 1, 1956): 556–558.

[2] Appleton, G. S., and White, P. G.: *Clostridium Botulinum* Type C. I. Studies on Laboratory Variables Affecting Toxin Production and Toxoid Evaluation. Am. J. Vet. Res., *18*, (Oct., 1957): 942–946.

[3] Kull, K. E., and Moberg, K.: Die Häufigkeit des Botulismus bei Futtervergiftungen des Nerzes in Schweden. Nord. Vet.-med., *4*, (1952): 771–779.

[4] Larsen, A. E., Nicholes, P. S., and Gebhardt, L. P.: Successful Immunization of Mink with a Toxoid Against *Clostridium Botulinum* Type C. Am. J. Vet. Res., *16*, (Oct., 1955): 573–575.

[5] Lewis, K. H., and Hill, E. V.: Practical Media and Control Measures for Producing Highly Toxic Cultures of *Clostridium Botulinum*, Type A. J. Bact., *53*, (1947): 213–230.

[6] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[7] Rice, Christine E.: A Preliminary Study of the Antigenic Activity of Mixtures of *Clostridium Botulinum* Toxoid Types A and B. Canad. J. Res., *25*, (1947): 181–187.

[8] Sterne, M., and Wentzel, L. M.: A New Method for the Large-Scale Production of High-Titre Botulinum Formol-Toxoid Types C and D. J. Immunol., *65*, (1950): 175–183.

XUM

Laboratory Evaluation of a Trivalent Toxoid for Botulism

GEORGE S. APPLETON, M.S., and PHILIP G. WHITE, Ph.D.

Madison, Wisconsin

IN A PREVIOUS PAPER, Appleton and White[2] reported on laboratory variables affecting toxin production and toxoid evaluation of *Clostridium botulinum* type C toxin and toxoid. These studies were initiated to provide a toxoid suitable for the immunization of mink against type C botulism. Since an outbreak of botulism in captive mink on a ranch in Colorado was reported to be caused by *Cl. botulinum* type A,[5] several investigators have studied the susceptibility of mink and other species to the toxin of both type A and *Cl. botulinum* type B.[3,4,9] It has been found that mink are highly susceptible to type C toxin but only slightly to moderately susceptible to the toxins of types A and B.

Botulism in mink usually has been caused by type C.[4,10] Outbreaks in other species, caused by types A, B, and C, are known. Man is highly susceptible to the toxins of types A and B,[13] while most avian species, as well as most ruminants, are susceptible to all three types.[13] Botulism has occurred in horses [1] and sheep.[13]

Although type C toxoids are produced commercially in the United States, immunization programs in many susceptible species would preferably utilize toxoids composed of at least three types (A, B, and C). This paper reports the results of laboratory studies on such a trivalent toxoid.

MATERIALS AND METHODS

Organisms.—Four type A strains, "Hall," X232-2AH-7, JTD-III, and a nonsporulating strain, and two type B strains, "Beans" and "Okra," were studied.* In a previous report,[2] it was shown that type C strain "South Africa" was highly satisfactory for toxoid production.

After verification of the serological type with specific antiserum† by toxin neutralization, strain "Hall" (type A) and strain "Okra" (type B) were selected for further study.

Medium.—The medium used to culture all three types consisted of proteose peptone (4%), enzymatic hydrolysate of casein (2%), yeast extract (2%), and dextrose (1%), with a final pH of 7.6 after autoclaving.

Toxins.—Whole culture toxins of the three types were produced in intussuscepted membranes (Visking) filled with physiological saline solution and immersed in the medium.[2,8,14]

Toxin Titration.—In all instances, titrations were conducted in healthy male white mice of one strain obtained from a single source. The mice weighed 15 to 25 Gm. All toxin dilutions were made in phosphate buffer (pH 6.5) containing 0.2 per cent gelatin.[6] Whole culture toxins were used for titrations. The $l.d._{50}$ end points were calculated by the method of Reed and Muench.[11]

Preparation of Toxoids.—Toxoids were prepared from five- to ten-day cultures of the three types grown at 37 C. by adding commercial formalin (0.5 to 0.6%) and reincubating at 37 C. for eight to 12 days. Toxoids were initially prepared by adding the formalin to undiluted cultures, but it was found that the cultures were highly resistant to detoxification in this state. Subsequent preparations, therefore, were diluted three- to fivefold before formolization. Complete detoxification was verified by parenteral injection of mice and guinea pigs (250 to 400 Gm.).

After detoxification, each of the three types was adsorbed separately onto aluminum complexes formed *in situ* by adding sterile potassium aluminum sulfate to a final concentration of 1.0 per cent of the original culture volume. The potassium aluminum sulfate was added under mild agitation, and the pH readjusted to 6.0 to 6.5 with sodium hydroxide. The adsorbed toxoid was allowed to settle in the refrigerator for 48 hours. The supernatant fluid from each type was decanted to the original volume of the harvested culture. After further adjusting the pH of each type to 6.0 to 6.5, they were pooled in equal volumes. The mixture, therefore, represented a 1:3 dilution of the original culture of each type. To study the effect of further dilution, 2 parts of a 0.5 per cent formolized physiological saline solution was added to 1 part of the mixture, which gave a 1:5 dilution of each type.

Immunization.—The relative toxicity of each of the three types (A, B, and C) was determined for mice, guinea pigs, and ferrets. Ferrets were found to be highly susceptible to type C toxin and only slightly susceptible to types A and B toxin, while

From the Research Division, American Scientific Laboratories, Inc., Madison, Wis.

* Strain "Hall" was provided by Dr. Carl Lamanna, Johns Hopkins University, Baltimore, Md.; strains X232-2AH-7 and JTD-III by Dr. J. Frederick Bell, Rocky Mountain Laboratory, Hamilton, Mont.; the nonsporulating strain and the two type B strains by Dr. George Wright, Fort Deterick, Frederick, Md.

† Supplied by Dr. Arden Killinger, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

mice and guinea pigs were almost equally susceptible to all three types. For reasons of economy and ease of handling and maintenance, the mouse was chosen for immunization studies. Healthy white mice were immunized by intraperitoneal injection of 0.5 ml. of toxoid and challenged 21 days later with lyophilized whole culture toxin.

RESULTS AND DISCUSSION

Mouse intraperitoneal titers of the whole culture toxins ranged from $10^{5.5}$ to $10^{6.5}$ $l.d._{50}$ per milliliter for all three types.

TABLE 1—Toxoid Dilutions Used for the Immunization of Mice

Type		Dilution	No. mice vaccinated
A		Undiluted	15
		1:3	15
		1:5	15
B		Undiluted	15
		1:3	15
		1:5	15
A-1/3	Combined	1:3	45
B-1/3		1:3	
C-1/3		1:3	
A-1/5	Combined	1:5	45
B-1/5		1:5	
C-1/5		1:5	
Saline-2/5			

Types A and B usually had a titer about 1 log higher than type C. For immunization of mice, dilution groups were prepared as shown (table 1).

The design of the challenge schedule was so arranged as to allow calculation of a "protection index" (*see* footnote, table 2). Type C was not studied as a monovalent preparation since numerous previous studies have been made with type C toxoid and its activity is well defined.[2] The results of challenge of mice vaccinated with various toxoid dilutions are given (table 2).

While the results reported here are limited, several observations of questionable significance may be made. Rice[12] observed that the antigenicity of type B toxoid was enhanced when mixed with type A toxoid. A reciprocal exalting effect of type A toxoid by type B toxoid was not evident in the work done by Rice or by the authors. In the work reported here, type B toxoid alone elicited a protection index of 250,000 at a dilution of 1:3. In combination with types A and C toxoids, at a dilution of 1:3, the protection index was greater than 250,000. Further, at a dilution of 1:5, type B toxoid alone gave a protection index of 5,000, whereas in combination with types A and C, at a dilution of 1:5, the protection index was 80,000.

Rice[12] attributed the enhanced immunizing efficacy of type B toxoid when mixed with type A toxoid to a nonspecific adjuvant effect. It is not possible to state, based on the results reported here, whether the apparent adjuvant effect on type B toxoid was because of type A, type C, or a combination of the two toxoid fractions.

The correlation between results, when mice were immunized with undiluted, 1:3, and 1:5 dilutions for types A and B monovalent preparations, was considered good. The difference in the protection index between either type, when tested alone at either 1:3 or 1:5 dilutions and when tested in combination at either 1:3 or 1:5 dilutions, is not considered significant. Because of the relatively small number of mice used, the difference between a protection index of 20,000 and one of 3,200 might be the result of the survival or death of only 1 or 2 mice. A variation of 0.2 to 0.3 of a

TABLE 2—The Resistance of Vaccinated Mice to Challenge with Clostridium Botulinum Types A, B, and C Toxins

		Results of challenge at 21 days postvaccination			
Toxoid type and dilution	Challenge toxin (type)	Log_{10} $l.d._{50}$ controls	Log_{10} $l.d._{50}$ vaccinees	Log difference	Protection index*
A-undiluted	A	6.0	1.0	5.0	100,000
A-1:3	A	6.0	1.4	4.6	40,000
A-1:5	A	6.0	1.7	4.3	20,000
B-undiluted	B	6.4	<1.0	>5.4	>250,000
B-1:3	B	6.4	1.0	5.4	250,000
B-1:5	B	6.4	2.7	3.7	5,000
ABC-1:3	A	6.0	1.7	4.3	20,000
ABC-1:3	B	6.4	<1.0	>5.4	>250,000
ABC-1:3	C	5.5	<1.0	>4.5	>32,000
ABC-1:5	A	6.0	2.5	3.5	3,200
ABC-1:5	B	6.4	1.5	4.9	80,000
ABC-1:5	C	5.5	<1.0	>4.5	>32,000

* The protection index is the antilog of the log difference between the $l.d._{50}$ of the control mice and the vaccinated mice. All mice were challenged intraperitoneally with 0.1 ml. of decimal toxin dilutions.

XUM

log in the titer of the challenge toxin from one evaluation to another also will reflect a variation of several thousands in the protection index results.

In initial toxoid preparations, with all three types, some difficulty was encountered in completely detoxifying the parent cultures. It was found, for example, that type A cultures, when detoxified as the original undiluted culture with 0.5 to 0.6 per cent formalin at 37 C. for 24 to 32 days, were still toxic when administered parenterally to guinea pigs. The same was true of type B cultures. Type C was more easily detoxified. Higher concentrations of formalin (0.8 to 1.0%) caused rapid destruction of the antigen at 37 C. Lower temperatures (room and refrigerator temperatures) were impractical because of the prolonged time necessary to completely detoxify the cultures (30 days to several weeks).

Suitable conditions for the detoxification of cultures and maintenance of high antigenicity in the toxoid were determined to be as follows: Type A, B, and C cultures were detoxified separately in a diluted state (diluted by a factor of 3 or 5) for ten to 14 days at 36 to 37 C. with 0.5 to 0.6 per cent commercial grade formalin.

SUMMARY

Several factors concerned with the preparation and evaluation of a trivalent toxoid containing equal parts of *Clostridium botulinum* types A, B, and C were studied.

1) Type A (strain Hall), type B (strain Okra), and type C (strain South Africa) were determined to be satisfactory strains for toxoid production.

2) The medium described was satisfactory for growth and toxin elaboration for all three types. Toxins for all three types were produced in intussuscepted membranes.

3) A suitable method for conversion of parent toxin to toxoid was described.

4) Mice were determined to be satisfactory experimental animals for the evaluation of types A, B, and C toxins and toxoids.

5) Trivalent toxoids, which served as satisfactory antigens in mice against large doses of toxin of all three types studied, were prepared by the methods and criteria described.

References

[1] Anon.: Equine Botulism in Spain. Vet. Bull., *26*, (April, 1956): 193.

[2] Appleton, G. S., and White, P. G.: *Clostridium Botulinum* Type C. I. Studies on Laboratory Variables Affecting Toxin Production and Toxoid Evaluation. Am. J. Vet. Res., *18*, (Oct., 1957): 942–946.

[3] Bell, J. F., Farrell, R. K., and Gorham, J. R.: A Polyvalent Toxoid for Botulism in Mink. Am. J. Vet. Res., *18*, (Jan., 1957): 167–170.

[4] Dinter, Z., and Kull, K. E.: Botulismus bein Nerz in Schweden. Nord. Vet.-med., *2*, (1950): 286–301.

[5] Hall, J. C., and Stiles, G. W.: An Outbreak of Botulism in Captive Mink on a Fur Farm in Colorado. J. Bact., *36*, (1938): 282.

[6] Lewis, K. H., and Hill, E. V.: Practical Media and Control Measures for Producing Highly Toxic Cultures of *Clostridium Botulinum*, Type A. J. Bact., *53*, (1947): 213–230.

[7] Moll, T., and Brandly, C. A.: Botulism in the Mouse, Mink, and Ferret with Special Reference to Susceptibility and Pathological Alterations. Am. J. Vet. Res., *12*, (Oct., 1951): 355–363.

[8] Polson, A., and Sterne, M.: Production of Potent Botulinum Toxins and Formol-Toxoids. Nature, *158*, (1946): 238–239.

[9] Quortrup, E. R., and Gorham, J. R.: Susceptibility of Furbearing Animals to the Toxins of *Clostridium Botulinum* Types A, B, C, and E. Am. J. Vet. Res., *10*, (July, 1949): 268–271.

[10] Quortrup, E. R., and Holt, A. L.: Case Report on Botulism Type C in Minks. J.A.V.M.A., *97*, (Aug., 1940): 167–168.

[11] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[12] Rice, C. E.: A Preliminary Study of the Antigenic Activity of Mixtures of *Clostridium Botulinum* Toxoid Types A and B. Canad. J. Res., *25*, (1947): 181–187.

[13] Smith, L. DS.: Introduction to the Pathogenic Anaerobes. 1st ed. University of Chicago Press, Chicago, Ill. (1955): 108–133.

[14] Sterne, M., and Wentzel, L. M.: A New Method for the Large Scale Production of a High-Titre Botulinum Formol-Toxoid Types C and D. J. Immunol., *65*, (1950): 175–183.

Studies on *Pasteurella Multocida*. IV. Serological Types from Species Other than Cattle and Swine

G. R. CARTER, D.V.Sc.

Guelph, Ontario

IN THE THIRD report[4] in this series, data were presented on the serological type, associated disease, and geographic source of strains of *Pasteurella multocida* recovered from cattle and swine. It was found that types A and D were the most prevalent varieties isolated in North America. Many bovine strains recovered from epizootic pasteurellosis (hemorrhagic septicemia) occurring in tropical and subtropical countries were type B.

It is the purpose of this report to give the results of the serological examination of a large number of strains of *Past. multocida* recovered from species other than cattle and swine.

MATERIALS AND METHODS

Strains of Pasteurella Multocida Examined.—These strains were submitted by laboratories in various parts of the world. Although most cultures were freshly isolated and had undergone few transfers, some were stock cultures of varying ages. The conventional criteria, as outlined by Merchant,[5] for the identification of *Past. multocida* were used.

Hemagglutination Test.—This test was carried out in the manner described previously.[3] It involved the adsorption of the specific capsular substance in the form of a saline solution extract to type O human erythrocytes and the addition of these treated cells to dilutions of the typing serums. A positive reaction consisted of agglutination of the erythrocytes, as shown by shaking after the tubes were left at room temperature for two hours. Four serum dilutions were used routinely. A positive reaction in the first two dilutions was required for type identification.

Acriflavine Test.—This test for the identification of morphological and antigenic characteristics of colonies of *Past. multocida* has been described.[3] As with other species, it has been shown that antigenic characteristics may change. The terms "mucoid" and "smooth" have been used in referring to colonial morphology, and "M," "S," and "S^R" in referring to antigenic characteristics. The term "S^R" designates the most important type of colony from the standpoint of serology. The S^R colonies are morphologically smooth but antigenically rough; *i.e.*, they do not possess the specific capsular substance.

All cultures examined serologically were subjected to the acriflavine test. The only reaction listed was the flocculent reaction obtained with cultures composed mainly of S^R colonies.

RESULTS

The type designations of 99 typable strains are listed (tables 1, 2). The cultures from a number of species are listed in table 1, while only avian strains are listed in table 2. The distribution of the different types for each species is shown (table 3).

Of 99 strains typed, 62 were type A, 14 were type B, and 23 were type D. The majority of the type B strains were from buffaloes. The type A and D strains were associated with a wide range of disease processes. Information relative to the associated disease was not available for a small number of cultures.

Many cultures were not typable by the hemagglutination test. These are referred to according to origin:

Cat Origin.—Of the 25 strains examined, 22 could not be unequivocally typed. Seventeen were antigenically rough, as determined by the acriflavine test. Ten were identified tentatively as type C but, because of cross reactions, it was thought advisable to defer positive identification. The majority of the cultures were recovered from the upper respiratory tracts of cats that had died from various causes.

Dog Origin.—Of the eight strains examined, seven were not typable; five of these seven were antigenically rough.

Fowl Origin.—Of 138 cultures, only 43 were clearly typable. Of the 95 nontypable cultures, 76 gave reactions with acriflavine that indicated antigenic roughness. The majority of the nontypable strains were recovered from chronic lesions that, in most instances, had no relation to acute

From the Department of Research, Ontario Veterinary College, Guelph.

This study was aided by a grant from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

The author thanks the laboratories that submitted strains of *Pasteurella multocida* for serological examination.

TABLE 1—Geographic Origin, Disease Process, and Serological Type of Strains of Pasteurella Multocida Recovered from Various Species

Species	No. cultures	Origin	Disease process	Serological type
Buffalo				
	2	France	Hem. sept.	B
	2	India	Hem. sept.	B
	3	Malaya	Hem. sept.	B
	3	Pakistan	Hem. sept.	B
	1	Turkey	Hem. sept.	B
	1	Philippines	.....	A
Cat				
	1	Massachusetts	.....	A
	1	France	.....	D
	1	Iowa	Wound	A
Deer				
	1	Holland	.....	B
	1	New York	.....	D
Dog				
	1	Alberta	Tooth abscess	D
Horse				
	1	Egypt	Hem. sept.	B
	1	Iowa	Lung	D
Man				
	1	Australia	Pneumonia	D
	1	Iowa	Bronchiectasis	D
	1	Alberta	.....	A
	1	Iowa	Sputum	D
	1	Iowa	Peritoneal fluid	D
	1	Ontario	Cat bite	A
	1	Ontario	Cat scratch	A
Mink				
	3	Alberta	.....	A
	1	Alberta	.....	D
Monkey				
	1	Iowa	.....	A
Mouse				
	1	Ontario	.....	D
	1	Alberta	Lung	D
Rabbit				
	2	Alberta	Pneumonia	D
	1	Illinois	Throat edema	A
	1	Ontario	Septicemia	D
	2	Ontario	Pneumonia	A
	1	Alberta	Skin abscess	A
	3	Ontario	Skin abscess	A
Sheep				
	2	Alberta	Pneumonia	D
	1	Alberta	Pneumonia	A
	1	Australia	Pneumonia	A
	1	Montana	Mastitis	A
	1	Montana	Mastitis	D
	2	Ontario	Pneumonia	A
	1	Ontario	Pneumonia	D
	1	Pakistan	Septicemia	B
	1	Ontario	.....	D
	1	California	.....	A

Hem. sept.= hemorrhagic septicemia.

fowl cholera. Some of the more frequent disease processes were sinusitis, tracheitis, keratoconjunctivitis, and wattle infection. Many strains probably were secondary invaders. Type A strains occurred most frequently in acute cholera.

Human Origin.—Of the ten strains collected, three were antigenically rough and nontypable.

Mink Origin.—Nine strains were examined, of which five were nontypable. Three of these were antigenically rough.

Mouse Origin.—Of a total of four cultures, two were nontypable.

Rabbit Origin.—Nineteen cultures were examined and nine were nontypable. Four strains were antigenically rough. The principal disease processes were abscesses and respiratory infection.

Sheep Origin.—A total of 27 strains was examined. Of the 15 cultures that were nontypable, only one was antigenically rough.

DISCUSSION

It is evident from the series of studies of which this is a part that there are three readily identifiable types of *Past. multocida; i.e.*, types A, B, and D. Types A and D are distributed widely, with regard to host range and disease process. As pointed out previously, type B cultures are the cause of epizootic hemorrhagic septicemia of cattle.

This and earlier studies indicated the existence of another major group, which was denoted "type C." There was evidence that this type occurred most frequently in dogs and cats. However, the hemagglutination test has not identified these strains clearly and, for this reason, many of the avian, canine, and feline strains that gave low-titer type C reactions were referred to as nontypable. That many of these strains constituted a separate group, immunologically, was demonstrated by a serum-protection test in mice, carried out as described by Roberts.[6] Bain[1] was of the opinion that many of the strains from dogs and cats may constitute a single group.

TABLE 2—Origin, Disease Process, and Serological Type of Strains of Pasteurella Multocida Recovered from Fowl

No. cultures	Origin	Disease	Serological type
9	Alberta	Chronic and acute cholera	A
2	Alberta	Chronic cholera	D
1	Australia	.....	A
1	California	.....	A
13	Formosa	Cholera	A
2	France	Cholera	A
1	Kenya	Cholera	A
4	Maine	Cholera	A
1	Minnesota	.....	A
2	Ontario	Cholera	D
5	Ontario	Acute cholera	A
2	Quebec	Acute cholera	A

The strains that would appear to be most pathogenic and were associated with definite disease processes were, for the most

part, typable. Studies with numerous antiserums would indicate that there are a number of types which occur less frequently.

It was observed that the colonies of a considerable number of nontypable cultures from various species gave a mucoid or stringy reaction in acriflavine. Their low virulence for mice, stringy growth in broth, and marked pleomorphism indicated a tendency toward roughness. These cultures should be distinguished from the strains of high virulence for mice, referred to perviously,[3] that gave the mucoid reaction in acriflavine.

Pasteurella multocida does not appear to be related immunologically to *Pasteurella hemolytica*. Neither the simple agglutination test nor the hemagglutination test described has shown any antigenic factors common to these two species.

TABLE 3—Distribution of Types in Species Other than Cattle and Swine

Species	Types			Not typable
	A	B	D	
Buffalo	1	11	0	0
Cat	2	0	1	22
Deer	0	1	1	0
Dog	0	0	1	7
Fowl	39	0	4	95
Horse	0	1	1	0
Man	3	0	4	3
Mink	3	0	1	5
Monkey	1	0	0	0
Mouse	0	0	2	2
Rabbit	7	0	3	9
Sheep	6	1	5	15
Totals	62	14	23	158

SUMMARY

Strains of *Pasteurella multocida* from various species, diseases, and geographic locations were examined serologically by the indirect hemagglutination test. Of 257 strains, only 99 were clearly typed. Of these, 62 strains were identified as type A, 14 as type B, and 23 as type D. All but two type B strains were recovered overseas from buffaloes with acute pasteurellosis (hemorrhagic septicemia). The type A and D strains were recovered from animals with a variety of disease processes, many of which were sporadic. Type A strains predominated as the cause of acute fowl cholera. The existence of a third large group or type was indicated but, because of inconclusive hemagglutination reactions, final identification was suspended.

References

[1] Bain, R. V. S.: Studies on Haemorrhagic Septicemia of Cattle. I. Naturally Acquired Immunity in Siamese Buffaloes. Brit. Vet. J., *110*, (1954): 481–484.

[2] Carter, G. R.: Studies on *Pasteurella Multocida*. I. A Hemagglutination Test for the Identification of Serological Types. Am. J. Vet. Res., *16*, (July, 1955): 481–484.

[3] Carter, G. R.: Studies on *Pasteurella Multocida*. II. Identification of Antigenic Characteristics and Colonial Variants. Am. J. Vet. Res., *18*, (Jan., 1957): 210–213.

[4] Carter, G. R.: Studies on *Pasteurella Multocida*. III. A Serological Survey of Bovine and Porcine Strains from Various Parts of the World. Am. J. Vet. Res., *18*, (April, 1957): 437–440.

[5] Merchant, I. A.: Veterinary Bacteriology and Virology. 4th ed. Iowa State College Press, Ames, 1950.

[6] Roberts, R. A.: An Immunological Study of *Pasteurella Septica*. J. Comp. Path. & Therap., *57*, (1947): 261–278.

A Serological Study on Some Strains of *Listeria Monocytogenes* Isolated in Michigan

JEANNETTE DONKER-VOET, D.V.M.

East Lansing, Michigan

THE ANTIGENIC characterization of *Listeria monocytogenes* dates from 1933, but the most important contributions were made by Paterson,[3,4] in 1939 and 1940.

Paterson investigated 54 strains and demonstrated the presence of both H and O antigens. Besides the common factor, B, he demonstrated three other factors, A, C, and D, in the flagellar system. In the O antigens, he distinguished three somatic groups, *i.e.*, a group with factors I, II, and (III), a group with factors II, IV, and (III), and a group with factors V and (III). The common factor, III, was regarded by him as unimportant. Paterson distinguished a total of four types:

Type 1: factors I, II, (III)	AB
Type 2: factors I, II, (III)	BD
Type 3: factors II, IV, (III)	AB
Type 4: factors V, (III)	ABC

Seeliger [6] distinguished two subtypes in the type 4 strains; he referred to these as subtypes 4a and 4b. These have a common factor, V, but Seeliger discussed no further details of the two subtypes in his book. He stated, however, that the known serotypes probably do not include all the antigenically distinct types which exist.

Donker-Voet [1,2] reported a subdivision of the type 4 strains into five subtypes, as follows:

Type 4a: (III), V, VII, IX	ABC
Type 4b: (III), V, VI	ABC
Type 4c: (III), V, VII	ABC
Type 4d: (III), (V), VI, VIII	ABC
Type 4e: (III), V, VI, VIII, IX	ABC

Factors III and V were found to occur irregularly in the strains. Types 4a and 4b correspond to the subtypes 4a and 4b of Seeliger.

MATERIALS AND METHODS

Most of the strains of *L. monocytogenes* used in this study were isolated from human beings, cattle, and sheep in Michigan.*

The technique used corresponds largely to that described by Paterson.[3,4] The nutrient medium used consisted of tryptose-phosphate broth and tryptose-phosphate agar (Difco).

A smooth, spreading (indicating motile organisms) colony from a pour-plate culture (0.65% agar), incubated for two days at room temperature, was transferred into tryptose-phosphate broth. The resulting growth was used to inoculate flasks of tryptose-phosphate broth which were incubated for two days at room temperature. Formalin then was added to a final concentration of 0.25 per cent. These suspensions were used both for the inoculation of rabbits and for H agglutination tests.

The original tryptose-phosphate broth culture also was used for the inoculation of Roux flasks containing tryptose-phosphate agar (2% agar). These bottles were incubated for two days at 37 C., and the resulting growth was removed with saline solution and then heated for two hours at 100 C. to destroy the flagella. The suspensions were centrifuged and resuspended in saline solution containing 0.25 per cent formalin. From these dense suspensions, suitable dilutions were made to provide antigens for the inoculation of rabbits and for O agglutination tests.

H and O antiserums were prepared against strains of each of the types and subtypes. Adult rabbits were injected intravenously by a schedule described by Paterson.[3,4] For the antibodies to the H antigen, the rabbits were injected five times at three-day intervals, with a rest period of ten days. This series was repeated at least once, several times in some animals, until an acceptable titer was obtained. The rabbits used for the production of antibodies against the O antigen were injected daily for six days, and a rest period of one week was permitted. This series was repeated at least once, and usually more, until a satisfactory titer was obtained. Levels of at least 1:1,280 for H antigens and at least 1:640 for O antigens were considered acceptable. The serum was not heated and was stored in the cold without a preservative.

The agglutination tests were set up in geometric series starting at a dilution of 1:20. Readings were made for the H agglutination tests after two hours in the water bath at 50 C. and for the O agglutination tests after three hours in the water bath at 45 C. and storage overnight in the incubator at 37 C.

The antigens used in the agglutination tests were adjusted at a density corresponding to tube 3 of the MacFarland nephelometer. The adjustments were made by direct observation, which

From the Department of Microbiology and Public Health, Michigan State University, East Lansing. The author is now at the Institute of Veterinary Bacteriology, Utrecht, Netherlands.

This study was made possible by an International Study Grant of the American Association of University Women and a Fulbright Travel Grant.

* The strains were collected by Dr. M. L. Gray while he was with the Department of Veterinary Pathology, Michigan State University, East Lansing.

TABLE 1—Strains of Listeria Monocytogenes Isolated from Human Beings

Strain (No.)	Place isolated	Source of culture	Age	Formula	Type
1176	Detroit	Brain	Adult	I, II, (III) AB	1a
967	Grand Rapids	Spinal fluid	Adult	I, II, (III) ABC	1b
2469	Muskegon	Spinal fluid	Adult	II, IV, (III) ABC	3b
455	Ann Arbor	Spinal fluid	18 months	V, VI, (III) ABC	4b
1782	Grand Rapids	Spinal fluid	Adult	V, VI, (III) ABC	4b
2311	Detroit	Blood	Adult	V, VI, (III) ABC	4b
2456	Detroit	Spinal fluid	Adult	V, VI, (III) ABC	4b
2470	Detroit	Spinal fluid	10 days	V, VI, (III) ABC	4b
2658	Detroit	Spinal fluid	Adult	V, VI, (III) ABC	4b
2749	Detroit	.....	Adult	V, VI, (III) ABC	4b
2454	Columbus	Spinal fluid	9 years	V, VI, (III) ABC	4b
1879	Georgia	Spinal fluid	Girl	V, VI, (III) ABC	4b
1809	Ann Arbor	Spinal fluid	3 weeks	V, VII, (III) ABC	4c
2659	Detroit	Spinal fluid	Adult	V, VII, (III) ABC	4c

may entail fairly extensive variations and, therefore, no absolute value can be attached to the figures obtained. The titer was recorded as the dilution in the last tube that gave at least 50 per cent agglutination.

Agglutinin-absorption tests were carried out with the serums to obtain complete removal of the homologous agglutinins. In this way, monospecific serums were prepared to ascertain which of the H antigens (A, C, D) and the O antigens (I, IV, V, VI, VII, VIII, IX) were present in each of the strains in our possession.

RESULTS

Among the cultures studied, there proved to be three groups of strains which have not been described previously. These have been designated "1b, "3b," and "4ab."

There were type 1 strains with the formula designated by Paterson [3,4] which contained factors I, II, (III) AB and were called "1a." The new type, 1b, possesses in addition the flagellar antigen, C, and has the formula, I, II, (III) ABC.

There was only one type 3 strain among the cultures investigated. This strain did not have the type 3 formula of Paterson [II, IV, (III) AB], but contained factors II, IV, (III) ABC, and was called "3b." Seeliger and Sulzbacher [7] mentioned two different type 3 strains, but gave no further details.

There was a group of type 4 strains with the factors V, VI, VII, IX, (III) ABC, which did not correspond to any described previously. This group has been designated "type 4ab." In some of these strains, the serological reaction with one of the factors, VI, VII, or IX, was weak.

Among the strains studied, 14 were isolated from human beings (table 1). Twelve were obtained in Michigan, one in Georgia, and one in Columbus, Ohio. All cultures were isolated from human beings with cases of meningitis.

The majority of the cultures (9) were found to be type 4b, two were 4c, and there was one representative each of types 1a, 1b, and 3b. Nine strains were from adults and five were from children.

There were 55 cultures of bovine origin, and most of them were isolated from the brains of cows with encephalitis (table 2). Six cultures were isolated from the livers of young calves, one strain was isolated from the cervix of a cow, and two strains were from the stomach contents of aborted fetuses.

The majority of the bovine strains (26) belonged to type 4b, four strains belonged to 4a, four to 4c, and nine strains were found to be type 4ab. Only 12 of the strains belonged to type 1 and, of these 12, six were isolated from young animals. Types 1a and 1b were equally divided.

TABLE 2—The Types and Antigenic Formulas of Strains of Listeria Monocytogenes Isolated from Various Tissues of Infected Cattle

	Types and antigenic formulas						
Source of culture	1a I, II, (III) AB	1b I, II, (III) ABC	4a V, VII, IX, (III) ABC	4b V, VI, (III) ABC	4c V, VII, (III) ABC	4ab V, VI,VII, IX, (III) ABC	Total
Brain	2	4	3	24	4	9	46
Liver	3	2	1	..	..	..	6
Fetus	1	..	..	1	..	..	2
Cervix	..	..	..	1	..	..	1
Total	6	6	4	26	4	9	55

XUM

TABLE 3—The Types and Antigenic Formulas of Strains of Listeria Monocytogenes Isolated from Sheep

Tissue	Types and antigenic formulas						
	1a I, II, (III) AB	1b I, II, (III) ABC	4a V, VII, IX, (III) ABC	4b V, VI, (III) ABC	4c V, VII, (III) ABC	4ab V, VI, VII, IX, (III) ABC	Total
Brain	18	18	....	30	3	5	74
Liver	1	....	....	....	....	....	1
Total	19	18	....	30	3	5	75

There were 74 strains isolated from the brains of sheep with encephalitis. One strain was obtained from the liver of a lamb (table 3).

Among the sheep strains, there were 37 strains belonging to type 1, 19 to type 1a, and 18 to type 1b. The remaining 38 strains belonged to type 4 and, of these strains, 30 were type 4b, three were 4c, and five were 4ab.

The results of the study of the antigenic structures of the 142 strains of *L. monocytogenes* isolated in Michigan are shown (table 4). Of these strains, 51 belonged to type 1, and 26 of these were type 1a and 25 were type 1b. One culture was a type 3b strain, and 90 strains were type 4 strains. The latter group was subdivided into four strains belonging to type 4a, 63 strains belonging to type 4b, nine strains belonging to type 4c, and 14 strains belonging to type 4ab.

TABLE 4—The Antigenic Types of the Cultures of Listeria Monocytogenes Isolated in Michigan

Source of culture	Antigenic types							
	1a	1b	3b	4a	4b	4c	4ab	Total
Man	1	1	1	....	7	2	....	12
Cattle	6	6	....	4	26	4	9	55
Sheep	19	18	....	....	30	3	5	75
Total	26	25	1	4	63	9	14	142

In addition, eight strains from cattle and sheep in Montana were studied. Five of these strains belonged to type 1a, one to type 4b, and two to type 4ab.

Nine strains isolated in Japan from sheep and goats also were investigated. Of these strains, eight belonged to type 4b and one strain, isolated from a goat, was type 1b.

DISCUSSION

Most of the strains of *L. monocytogenes* isolated in Michigan belonged to type 4. The most common of the type 4 strains is the subtype 4b. These results differ from those found in Germany. Potel [5] reported from East Germany that, of 156 human strains, 147 were type 1. Seeliger,[6] in West Germany, wrote that "Type 1 is known to be prevalent and the other types are relatively rare in Germany." In Holland, types 1 and 4 are about equally divided.

The percentage of type 1 strains was higher in sheep than it was in cows and human beings. Among the type 1 strains, type 1a [I, II, (III) AB] and type 1b [I, II, (III) ABC] were about equally divided. Although type 1b has been found in Holland and in Switzerland, this type is rare in Europe.

There was only one type 3 among the strains studied here. Dr. Seeliger has one type 3 strain in his collection which was isolated in the United States. This strain has the same formula [II, IV, (III) ABC] as that found for the single type 3 strain reported here and it is, presumably, the same culture.

Among the cultures, there was a group which contained the factors V, VI, VII, and IX. This group was called "4ab." In some of these strains, there was only a weak reaction with the serums specific for the factors VI, VII, or IX.

The cultures studied were isolated principally from man and animals in Michigan and may not be entirely representative of the occurrence of the various antigenic types in the United States.

There seems to be little correlation between the antigenic type and the species of the animal from which the culture was obtained or the region of Michigan in which the animals, or man, were found.

SUMMARY

The antigenic types of 142 strains of *Listeria monocytogenes* isolated in Michigan were studied. Of these strains, 51 belonged to type 1. The strains were subdivided into 26 strains with the formula, I, II, (III) AB, and 25 strains with the

XUM

formula, I, II, (III) ABC. These were designated "1a" and "1b."

Type 2 was not found among the cultures investigated.

Only one strain belonged to type 3. It had the formula, II, IV, (III) ABC, and was called "3b."

The remaining 90 strains belonged to type 4. These were subdivided into four strains belonging to type 4a [formula V, VII, IX, (III) ABC], 63 strain belonging to type 4b [formula V, VI, (III) ABC], nine strains belonging to type 4c [formula V, VII, (III) ABC], and 14 strains called "4ab" [formula V, VI, VII, IX, (III) ABC].

References

[1] Donker-Voet, J.: Serological Studies on Some Strains of *Listeria Monocytogenes*. Tijdschr. Diergeneesk., *82*, (1957): 341–350.

[2] Donker-Voet, J.: Serological Studies on Some Strains of *Listeria Monocytogenes* Especially Type IV Strains. Zentralbl. f. Vet.-Med., *5*, (1958): 37–44.

[3] Paterson, J. S.: The Antigenic Structure of Organisms of the Genus Listerella. J. Path. & Bact., *51*, (1940): 427–436.

[4] Paterson, J. S.: Flagellar Antigens of Oganisms of the Genus Listerella. J. Path. & Bact., *48*, (1939): 25–32.

[5] Potel, J.: Zum gegenwärtigen Stand der Listerioseforschung. Wiss. Ztschr. Univ. Halle, Math.-Nat. Abt., *4*, (1957): 311–334.

[6] Seeliger, H. P. R.: Listerioses. Johann Ambrosius Barth, Leipzig, Germany, 1955.

[7] Seeliger, H. P. R., and Sulzbacher, F.: Antigenic Relationships Between *Listeria Monocytogenes* and *Staphylococcus Aureus*. Canad. J. Microbiol., *2*, (1956): 220–231.

XUM

A Pooled Human Serum as a Standard and the Effects of Freezing and Thawing on the Electrophoretic Pattern of Baby Pig Serum

ROBERT R. PENSINGER, M.S.; ELWOOD F. REBER, Ph.D.; ETEM ERSOY, Ph.D.; HORACE W. NORTON, Ph.D.

Urbana, Illinois

EXTENSIVE STUDIES on the development of procedures and techniques for paper electrophoretic separation of serum components have been reported.[2, 4, 5, 9]

Henry *et al.*[4] described the stability of serum at room and refrigerator temperatures, but did not consider the effects of freezing and thawing. Mathews and Buthala [6] reported that no significant alteration of the protein pattern resulted from repeated freezing and thawing. Moore *et al.*[7, 8] stated that a comparison of the results of analysis of fresh human serum with those of serum that had been stored in a frozen state was not justified. They reported that freezing and thawing adult human serum caused a decrease in albumin and a corresponding increase in *gamma* globulin.

However, the following problems arose in this laboratory which were not answered in available literature: (1) the establishment of a standard for comparison of pig serum from one analysis to another; (2) adequate resolution of protein components; and (3) the effect of freezing and thawing on the protein components of pig serum. The investigation of these problems is the subject of this paper.

MATERIALS AND METHODS

A modification of the procedure outlined elsewhere [1] was used for the analyses. A Durrum type of electrophoresis cell was used throughout this investigation.* It accommodates eight paper strips during each run. A veronal buffer of pH 8.6 at an ionic strength of 0.075 was used. Strips were dyed for six hours in a bromphenol blue dye bath. The strips were scanned with the Analytrol recording scanner and integrator.* All data are presented as relative percentages of total serum proteins.

No satisfactory standard was known for pig serum analyses. In experiment 1, the following materials were studied to determine their value as standards: bovine albumin;† bovine plasma *delta* albumin, 5 per cent solution;† porcine plasma albumin, 5 per cent solution;† and porcine *gamma* globulin, 10 per cent solution.† In addition, a mixture of 0.1 ml. porcine *gamma* globulin, 0.1 ml. bovine albumin, and 0.2 ml. distilled water was used. Later, standard human serum (Versatol**) was used.

The standard human serum was applied to one of eight paper strips in 34 electrophoretic separations to determine if there were variations in results that could be attributed to the operator's technique and the accuracy of the electrophoresis cell and the scanner and integrator. One operator made 14 sets of separations at 5 ma. for 16 hours; another operator made 20 sets of separations at 21 ma. for five hours.

Two experiments were conducted to determine the effects of freezing and thawing. The standard was the diluted mixture of porcine *gamma* globulin, bovine albumin, and distilled water. Heart blood samples were taken from pigs. Clotted blood was centrifuged and the serum was removed with a capillary pipette. Fresh serum was temporarily stored in a refrigerator and analyzed six hours later. The remainder of each sample was frozen and held more than one week at −18 C. The samples were then thawed in water not over 37 C., mixed, and reanalyzed. All the pig serum samples were analyzed at 5 ma. for 16 hours.

Experiment 2 was an analysis of serum obtained from 2 Hampshire pigs at 22, 24, 31, 45, and 52 days of age. Each serum sample was analyzed in triplicate, before and after freezing.

Experiment 3 was an analysis of serum obtained from 6 Hampshire pigs at 4, 10, 12, 17, 19, 24, 26, and 31 days of age. In this experiment, each sample was analyzed once before and once after freezing.

From the Department of Veterinary Physiology and Pharmacology, University of Illinois, and the Illinois Agricultural Experiment Station, Urbana, as collaborators under the North Central Regional Agricultural Experiment Station cooperative research project entitled "Death Losses in Young Pigs." Dr. Ersoy is now at Veteriner Fakultesi, Ankara, Turkey.

The authors thank Mr. Winfield Samuelson, Department of Veterinary Physiology and Pharmacology, University of Illinois, for technical assistance.

* Manufactured by Beckman Instruments Inc., Spinco Division, Palo Alto, Calif.

† Supplied by Armour Veterinary Laboratories, Kansas City, Kan.

** Versatol is prepared from freeze-dried human serum to exact standards, with the electrophoretic pattern of normal human serum, and is produced by Warner-Chilcott Laboratories, Morris Plains, N.J.

TABLE 1—The Effect of Time and Amperage on the Average Relative Percentages of Protein Components of the Standard Human Serum

Time (hours)	Current (ma.)	No. samples	Albumin	Globulins		
				Alpha	*Beta*	*Gamma*
16	5	14	60 ± 5.5*	12 ± 2.4	11 ± 2.4	17 ± 2.6
5	21	20	58 ± 6.8	14 ± 3.6	11 ± 2.6	18 ± 3.1

* Average relative percentage plus or minus the standard deviation of the individual samples.

RESULTS AND DISCUSSION

The commercial protein solutions were considered individually. Two of the solutions were extracted from plasma. Plasma albumin was unsatisfactory because of its albumin "tail." The tail represents a fraction of globulins or other impurities that migrate more slowly than the albumin, and is a characteristic pattern that might be expected with impure albumin. Bovine plasma *delta* albumin left a substance at the point of application on the paper. Bovine albumin and porcine *gamma* globulin standards were useful in comparing the separation of these protein components in pig serum. These standard solutions had known concentrations of albumin or globulin. A diluted mixture of the two standards was found superior, primarily because the standard solutions were too concentrated to be analyzed. However, not all the fractions observed in pig serum were represented in these standards.

The standard human serum, which compared favorably with the protein fractions of pig serum, separated into the four basic components: *gamma* globulin, *beta* globulin, *alpha* globulin, and albumin. *Alpha* globulin is sometimes regarded as consisting of two components, *alpha*-1 and *alpha*-2. This separation was not considered, because these components did not both consistently appear in the standard human serum or baby pig serum analyses.

The effects of variation of time and amperage on protein separation of pooled human serum are presented (table 1). Samples were electrophoretically separated for five or 16 hours. There appeared to be little difference between the two time intervals.

The next problem was the determination of suitable conditions for the resolution of protein components of serum. Pooled human serum components appeared to be adequately resolved under the condition of amperage and length of time of separation described. In addition, optimum conditions for analyzing baby pig serum were found. Electrophoretic separation at 5 ma. for 16 hours was satisfactory for resolving

TABLE 2—Relative Percentages of the Protein Fractions of Fresh Porcine Serum and Serum Thawed After Freezing

Item	Age* (days)	Mean percentage		Change after freezing†	
		Fresh	Frozen	Range	Average
Albumin	22	41	45	0 to +8	+4
	24	42	44	−5 to +8	+2
	31	48	43	−9 to −1	−5
	45	45	39	−12 to −1	−6
	52	38	35	−9 to +4	−3
Alpha globulin	22	24	21	−1 to −7	−3
	24	22	23	−1 to +7	+1
	31	21	24	−1 to +5	+3
	45	26	27	−5 to +8	+1
	52	26	26	−4 to +4	0
Beta globulin	22	20	19	−4 to +2	−1
	24	19	18	−4 to +4	−1
	31	18	18	−2 to +1	0
	45	18	20	0 to +5	+2
	52	19	21	−2 to +6	+2
Gamma globulin	22	15	14	−2 to +1	−1
	24	17	14	−5 to +4	−3
	31	12	14	−4 to +4	+2
	45	12	14	−1 to +5	+2
	52	17	18	−2 to +3	+1

* Samples were collected from 2 pigs at each age, and each sample was analyzed in triplicate when fresh and after freezing. † The standard error of the mean for albumin was ± 5; *alpha* globulin, ± 4; *beta* globulin, ± 3; and *gamma* globulin ± 3.

XUM

the four basic protein components of young pig serum.

The average relative percentages of the albumin and globulin fractions in pig serum before and after freezing, as determined in experiment 2, are shown (table 2). Two animals had been used in this initial experiment in which the problem of freezing and thawing was investigated. There seemed to be definite changes with freezing and thawing. The direction of change, increase or decrease, varied with age. When the albumin data were analyzed statistically, a significant interaction (1% level) of age and freezing was found. However, it was decided that the age relationship was beyond the scope of this paper. It is, therefore, our purpose to determine whether changes occur with respect to the protein components when serum is frozen.

As the protein components are reported in relative percentages of the total protein, the total globulin percentage is 100 minus the percentage of albumin. *Alpha* and *gamma* globulin showed most of the changes which occurred in the globulin fractions. There appeared to be less effect on the relative percentages of *beta* globulin due to freezing and thawing.

The relative percentages of the protein fractions in pig serum, obtained in experiment 3, are presented (table 3). Freezing and thawing of these pig serums caused the mean relative percentages of albumin to increase, except at one age. Samples from 4-day-old pigs were the exception to this change. The increase in the relative percentage of albumin was from 3.12 to 7.54, significant at the 99.0 per cent confidence level.

Globulin fractions generally tended to decrease with freezing. Relative percentages of *beta* and *gamma* globulin decreased more than did that of the *alpha* globulin as a result of freezing and thawing.

On the basis of the findings with baby pig serum, as well as those of Moore *et al.*[8] for adult human serum, it is apparent that freezing and thawing have an effect on the relative percentages of serum components. With human adult serum, Moore *et al.*[8] reported a decrease in the albumin fraction

TABLE 3—Analysis of the Protein Fractions of Fresh Porcine Serum and Serum Thawed After Freezing

Item	Age* (days)	Mean percentage		Change after freezing†	
		Fresh	Frozen	Range	Average
Albumin	4	15	14	−8 to +2	−1.5
	10	27	28	−1 to +3	+0.8
	12	29	35	0 to +14	+5.8
	17	32	38	+2 to +11	+6.0
	19	32	40	+6 to +11	+8.3
	24	31	46	+8 to +19	+14.7
	26	41	46	+1 to +9	+4.7
	31	47	50	−4 to +6	+3.3
Alpha globulin	4	26	23	−9 to +3	−2.3
	10	26	32	0 to +12	+6.5
	12	28	28	−3 to +5	+0.2
	17	29	28	−5 to +6	−0.5
	19	30	29	−6 to +4	−1.0
	24	28	26	−6 to +2	−1.7
	26	26	26	−3 to +4	−0.3
	31	25	25	−3 to +7	+0.5
Beta globulin	4	25	22	−6 to +2	−2.3
	10	22	19	−5 to −1	−3.5
	12	22	21	−7 to +2	−0.8
	17	25	22	−6 to −1	−3.5
	19	25	21	−7 to +2	−4.2
	24	27	20	−1 to −10	−7.5
	26	21	18	−5 to −1	−2.5
	31	18	16	−6 to +4	−1.5
Gamma globulin	4	34	41	−3 to +21	+6.8
	10	24	21	−10 to +2	−3.7
	12	20	16	−10 to −2	−4.7
	17	14	12	−6 to 0	−2.7
	19	13	10	−5 to −2	−3.7
	24	13	9	−3 to −7	−4.7
	26	12	10	−7 to +2	−1.5
	31	11	9	−5 to +2	−2.0

* Samples were collected from 6 pigs at each age and analyzed fresh and after freezing. † The standard error of the mean for albumin was ± 1.45; *alpha* globulin, ± 1.48; *beta* globulin, ±1.27; and *gamma* globulin, ± 1.62.

and an increase in the *gamma* globulin fraction. The same changes occurred in pigs 45 and 52 days old. It is suggested that this effect probably persists in pig serum taken from animals over 1 month old. However, the changes are opposite to those observed in samples from pigs less than 1 month old. Additional investigation is needed to determine the effect of age on changes due to freezing and thawing.

The studies of human serum by Moore *et al.*[8] and the studies reported here indicated that frozen samples are not equivalent to fresh samples. Disagreements between workers may result from differences in technique.

Moore *et al.*[8] suggested that drastic temperature changes cause protein denaturation. Meryman,[7] in a report on the mechanics of freezing in living cells and tissues, stated: "The principal cause of injury from slow freezing is not the physiological presence of extracellular ice crystals, but the denaturation increased by dehydration resulting from the incorporation of all free water into ice."

SUMMARY

A pooled human serum is recommended as a standard for pig serum analyses. It may also be used for checking technique, equipment, and reagents. Resolution of the standard components is adequate at 5 ma. constant current for 16 hours, or 21 ma. for five hours. Pig serum was resolved with 5 ma. constant current for 16 hours. Because freezing and thawing significantly altered the protein pattern of baby pig serum, fresh serum should be analyzed in preference to frozen serum.

References

[1] Anon.: Spinco Model R. Paper Electrophoresis Apparatus Operating Instructions. Form OIR-1. Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., 1956.

[2] Block, R. J., Durrum, E. L., and Zweig, G. E.: A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York, N.Y., 1955.

[3] Foster, J. F., Friedell, R. W., Catron, D., and Dieckman, M. R.: Electrophoretic Studies on Swine. III. Composition of Baby Pig Plasma and Sows Whey During Lactation. Arch. Biochem., *31*, (1950): 104–112.

[4] Henry, R. J., Golub, O. J., and Sobel, C.: Some of the Variables Involved in the Fractionation of Serum Proteins by Paper Electrophoresis. Clin. Chem., *3*, (1957): 49–64.

[5] Lederer, M.: An Introduction to Paper Electrophoresis and Related Methods. Elsevier Press, Princeton, N.J., 1955.

[6] Mathews, J., and Buthala, D. A.: Effect of Repeated Freezing and Thawing on the Electrophoretic Pattern of Swine Serum. Am. J. Vet. Res., *17*, (July, 1956): 485–486.

[7] Meryman, H. T.: Mechanics of Freezing in Living Cells and Tissues. Science, *124*, (1956): 515–521.

[8] Moore, D. H., Roberts, J. B., Costello, M., and Shonberger, T. W.: Factors Influencing the Electrophoretic Analysis of Human Serum. J. Biol. Chem., *180*, (1949): 1147–1158.

[9] Wolstenholme, G. E. W., and Miller, E. C. P., ed.: Paper Electrophoresis. Ciba Foundation Symposium. Little, Brown, and Co., Boston, Mass., 1956.

The Body Fluids of Ruminants and Their Simultaneous Determination

ELLIOTT L. HIX, Ph.D.; G. K. L. UNDERBJERG, D.V.M., Ph.D.; J. S. HUGHES, Ph.D.

Manhattan, Kansas

WATER is the largest single component of the mammalian body and one of the essential items of the diet. Its normal distribution within the body is less appreciated. The development of simple and reliable procedures for indirect *in vivo* determination of the volume of the various fluid phases of the body enable one to separate the total body water (TBW) into blood plasma water (PW),[13] extracellular water (ECW),[8,15] interstitial water (ISW), and intracellular water (ICW).[1]

Quantitative determination of TBW using tritiated (H^3) water[32] indicates that, while water is an extremely variable component of the bodies of mammals, the water and nitrogen content of the fat-free lean body mass is relatively constant.[33] The concept has developed that the mammalian body consists of a variable quantity of fat and water, but of a lean body mass constant in gross chemical composition. This constancy is regarded as one of the important biological constants.[36]

With the introduction of antipyrine by Brodie *et al.*[2] for the *in vivo* determination of TBW, an inverse relationship between body fat and water has been further qualified and quantitated in man,[38,39] cattle,[25] and swine.[24] These studies have revealed the accuracy and application of the dilution method of analysis (antipyrine space) to studies of body water and body composition.

Day-to-day fluid shifts among the several fluid compartments of the body, however, render separate analysis of their volumes impractical in many applications. Therefore, a procedure for the simultaneous determination of ECW and TBW is desirable and preferable for determining these fluid shifts under any circumstance which may create special problems and aberrations in fluid balance.

MATERIALS AND METHODS

The experimental animals were 24 western-type black face wether sheep. Before instituting the mineral balance studies (Na, K, and Cl) reported earlier,[19] the volumes of the body fluids were determined to establish a reference parameter (normal) for the type and species. Plasma water, ECW, and TBW analyses were repeated in 18 of these animals, ten days after the initial analyses. Other experimental animals were 3 wethers hydrated by high dietary salt (NaCl) intake, 3 wethers dehydrated by high dietary potassium bicarbonate ($KHCO_3$) intake, 9 goats of mixed breeding, and 5 Shorthorn calves, 8 to 9 months old, of close inbreeding. The sheep and calves were on balanced rations, while the goats were on a roughage maintenance ration and were extremely lean as judged from somatotype. Due to variation in volume of rumen content, all animals were fasted for 24 hours and given no water eight to 12 hours before fluid volume determinations. This adjusted body weight has been observed to approach the empty body weight consistently.[23] The sheep were sheared closely to obtain a wool-free weight.

Extracellular water and TBW were determined simultaneously in all animals. Plasma water volume was determined only in the sheep and goats, and at the same time as the other determinations.

Determination of Plasma Volume.—Plasma water volume was determined by the method of Kennedy and Millikan,[20] using the blue dye T-1824 (Evans blue), modified as follows to avoid micromethods and to adapt it to the Evelyn photoelectric colorimeter.

A 1.0 per cent solution of T-1824 was prepared and filtered to remove particulate insolubles. Five milliliters was injected into the jugular veins of the sheep or goats from an accurately calibrated syringe. After ten minutes, a blood sample was drawn from the vein opposite to that injected, the sample was centrifuged, and the plasma separated in heparinized tubes.

To 2.0 ml. of plasma in a test tube, 8.0 ml. of distilled water was added, and the density of this 20 per cent plasma sample was determined at $\lambda = 620$ mμ. A 20 per cent plasma sample free of dye

Contributions No. 201, from the Department of Animal Husbandry; No. 136, Department of Veterinary Medicine; and No. 501, Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan.

Drs. Underbjerg and Hughes are with the Departments of Physiology and Chemistry, respectively, Kansas State College, Manhattan. Dr. Hix is now with the Department of Physiology and Pharmacology, Kirksville College of Osteopathy and Surgery, Kirksville, Mo.

was used as a blank. The Lambert-Beer law is effective over a plasma dye-concentration range of 8 to 40 μg. per milliliter with either 10 or 20 per cent plasma samples, and only one reference standard of 20 μg. of dye per milliliter of plasma is necessary.

Simultaneous Determination of Extracellular and Total Body Water.—Stock solutions of 20 per cent antipyrine (1-phenyl-2,3-dimethylpyrazolone-5) and 20 per cent sodium thiocyanate (NaSCN) were prepared in aqueous solution. Equal volumes of each stock solution were mixed to give a solution of 10 per cent antipyrine and 10 per cent NaSCN for injection. A sufficient volume was injected intravenously to give a serum concentration of 30 to 40 μg. of antipyrine after equilibrium was established with the body fluids. The normal ratio of ECW to TBW will insure a thiocyanate ($^-$SNC) concentration in serum of 9 to 12 mg./100 ml. For the sheep and goat, 10 ml. is sufficient while 30 to 40 ml. is adequate for cattle weighing 170 kg. Blood samples were taken from the jugular vein opposite to that injected at postinjection intervals of two, three, four, and five hours. The sample was centrifuged and serum or plasma (heparinized) separated for analysis.

Antipyrine and Thiocyanate Analysis.—Two milliliters of serum was placed in a 50-ml. capacity glass-stoppered centrifuge bottle with a tapered bottom, and 1 ml. of 0.5 N sodium hydroxide was added. Twenty milliliters of redistilled chloroform was added and the bottle was sealed securely and shaken for 20 minutes. The bottle then was centrifuged at 4,000 r.p.m. for 15 minutes and the solvent phase quantitatively drawn off through a 5-inch hypodermic needle attached to syringe of 20-ml. capacity. Antipyrine resides in the solvent phase and, as it interferes with $^-$SCN analysis, its removal must be quantitative. The aqueous phase remaining in the centrifuge bottle was temporarily set aside.

A 15.0-ml. aliquot of the solvent phase containing the antipyrine was transferred to a 50.0-ml. beaker and evaporated to dryness at room temperature. The residue was taken up in 5.0 ml. of 0.07 N sulfuric acid, and 0.1 ml. of 0.2 per cent sodium nitrite ($NaNO_2$) solution was added. After 25 minutes, the solution was transferred to silica cells and the density determined in the ultraviolet at $\lambda = 350$ mμ, using a Beckman model DU spectrophotometer. A blank for the zero setting consisted of 5.0 ml. of 0.07 N sulfuric acid to which had been added 0.1 ml. of 0.2 per cent sodium nitrite. The density of a standard antipyrine solution containing 10 μg. per milliliter was determined along with the samples. Preferably, 2.0 ml. of the standard anti-

TABLE 1—The Fluid Compartments in Ruminants and Calculated Body Fat

Species and condition	Body weight (kg.)	Blood plasma water (% body wt.)	Extracellular water (% body wt.)	Interstitial water (% body wt.)	Total body water (% body wt.)	Calculated fat (% body wt.)
NORMAL ANIMALS (1952 to 1953)						
Sheep (18)*	47.5 (45.0-54.0)	5.8 (5.5-6.0)	30.0 (29.5-30.2)	24.2 (23.0-29.0)	56.2 (53.0-59.0)	22.6 (19.0-27.0)
SHEEP WITH MILD HYDRATION (4/21/52)						
19	40.6	6.3	32.1	25.8	55.3	.....
26	40.4	6.5	32.8	25.3	56.0	.....
56	42.3	6.1	31.8	25.7	56.3	.....
SHEEP WITH MILD DEHYDRATION (4/21/52)						
194	36.6	5.5	28.5	23.0	56.2	.....
220	38.2	5.8	29.2	23.4	54.0	.....
228	41.4	5.3	28.8	23.5	59.8	.....
SHEEP WITH SEVERE DEHYDRATION (4/9/53)						
194	53.4	4.2	22.4	18.2	50.2	.....
228	52.0	4.5	22.7	18.2	48.4	.....
GOATS, NORMAL (9/30/52)						
24	39.1	6.7	29.9	23.3	70.0	3.6
25	55.0	5.4	28.9	23.5	68.8	5.2
26	71.8	5.9	29.3	23.4	71.0	2.3
27	36.4	5.3	30.1	24.8	68.3	5.9
78 †	37.7	8.1	31.0	25.5	67.0	.....
80	40.5	6.0	29.7	23.7	67.5	7.1
81	44.3	6.6	30.2	23.6	69.2	4.7
82	44.3	5.6	30.6	25.0	69.5	4.3
84 **	46.8	8.9	29.5	21.4	72.2	.....
Mean		5.9	29.8	23.9	69.1	4.7
CATTLE, NORMAL (11/7/52)						
4	170.4	.....	29.3	.....	65.6	9.6
39	166.8	.....	29.7	.....	63.3	12.8
49	158.6	.....	27.6	.....	60.1	17.3
61	169.1	.....	28.3	.....	65.7	9.6
92	160.4	.....	27.9	.....	61.0	16.0
Mean		.....	28.5	.....	63.1	13.0

* Normal average for 18 animals with ranges in parentheses; †animal pregnant; blood plasma water on two occasions was 7.8 and 8.3. ** Animal died 24 hours after the determination. Necropsy revealed extensive hypostatic congestion of lungs and hemorrhagic effusions. The dye (T-1824) was observed in lymph nodes, indicating extensive vascular leakage.

XUM

pyrine solution is carried through the analytical procedure in triplicate. Antipyrine was quantitated in duplicate samples by the following equation:

$$\frac{D_{sample}}{D_{standard}} \times \frac{50.0}{1.5} = \mu g. \text{ antipyrine/ml.}$$

To the aqueous phase containing the $^-$SNC, 5.0 ml. of 10 per cent trichloroacetic acid was added to the centrifuge bottle, shaking it to precipitate the proteins. The sample was filtered and 4.0 ml. of the filtrate was transferred to a test tube. Four milliliters of distilled water and 2.0 ml. of ferric nitrate reagent* were added. The density was determined at $\lambda = 490$ mμ with a spectrophotometer (Coleman model 14). A blank consisting of 2.5 ml. of trichloroacetic acid, 5.5 ml. of water, and 2.0 ml. of ferric nitrate reagent was used for the zero setting. A single sodium thiocyanate standard containing 0.10 mg. in 10.0 ml. was used, and the unknown samples were quantitated as follows:

$$\frac{D_{sample}}{D_{standard}} \times 0.10 = \text{mg. } ^-SCN/\text{ml.}$$

The distribution volume of antipyrine and $^-$SCN was determined by the following relationship:

$$\text{Distribution volume} = \frac{\text{mg. injected}}{\frac{\text{mg./100 ml. serum} \times 10}{0.935}}$$

The factor, 0.935, accounts for a serum protein content of 6.5 Gm./100 ml.† Therefore, the distribution volume is in terms of liters of water as required.

The serum concentration of antipyrine at zero time (t_o) was determined by plotting the serum levels of the serial samples on semilogarithmic paper and extrapolating the exponential disappearance time-concentration curve (straight line) back to t_o. This is valid on the assumption that the serum concentration at t_o represents the concentration at the time of injection if instantaneous mixing had occurred and none of the antipyrine had been metabolized.

RESULTS

The analytical scheme described is an adaptation of the methods of Brodie *et al.*,[2] for antipyrine, and Crandall and Anderson,[5] for $^-$SCN, to facilitate the simultaneous determination of TBW and ECW. The presence of antipyrine in plasma interferes with the analysis of $^-$SCN and results in erroneously high quantitative values. Recovery of antipyrine from serum was good at 96 to 98 per cent, but $^-$SCN recovery included an artifact in highly concentrated serums. In normal serum $^-$SCN concentrations (10 mg./100 ml.), recovery was 99 to 101 per cent. High serum $^-$SCN levels should be avoided, both for accurate analysis and to prevent possible toxic reactions.[12]

Thiocyanate reached equilibrium with the extracellular fluids in the sheep and goats 60 to 90 minutes postinjection. In these ruminants, $^-$SCN was metabolized slowly [11] or excreted [18] at 4 to 5 per cent of the serum level per hour, while cattle showed a consistent serum level of $^-$SCN for six hours postinjection. Antipyrine was metabolized by cattle at the rate of 26 per cent of the injected dose per hour, but at a higher rate by the sheep and goat (28–30 %). In the goats, high initial serum levels of antipyrine (40–60 μg./ml.) were found advantageous. No signs of toxicosis from antipyrine or $^-$SCN were observed.

Early in our studies of the fluid compartments in ruminants, the sheep was used in developing procedures and data on 18 normal sheep were collected (table 1). In the normally hydrated sheep, the ECW ($^-$SCN space) was consistent at 30.0 per cent of the adjusted sheared body weight, and showed an extremely narrow range of 29.5 to 30.2 per cent. The TBW (antipyrine space) also was relatively limited in range (53.0–59.0% body weight), indicating that the experimental animals were uniform in gross composition or exhibited body fat contents of 19.0 to 27.0 per cent of body weight.

Body water analyses in 1 aged and debilitated ram revealed an ECW volume of 35.0

TABLE 2—Body Composition of a Wether as Determined from the Analysis of the Various Body Fluid Compartments

Body constituents	Kilograms	Percentage of body weight
OBSERVED DATA *		
Body weight (wool-free)	47.50	100.0
Body water compartments		
Total body water (antipyrine space)	26.68	56.2
Extracellular water ($^-$SCN space)	14.25	30.0
Plasma water (T-1824 space)	2.75	5.8
DERIVED DATA		
Interstitial water (ECW—PW)	11.50	24.2
Intracellular water (TBW—ECW)	12.43	26.2
Body fat (100 — % TBW/0.726)	10.73	22.6
Lean body mass (body wt.— fat)		
Dry matter (body wt.— TBW)	20.82	43.8
Organic nonfat (dry matter — fat)	10.09	21.2

* The quantitative values for the several body fluid components of the total body water were determined *in vivo* by dilution analysis. The lean body mass represents the physiologically active "tissue mass." It is this active tissue mass which determines basal heat production.

* Ferric nitrate reagent is prepared by dissolving 25 Gm. of ferric nitrate [$Fe(NO_3)_3 \cdot 9$ HOH] in 500.0 ml. of water and adding 12.5 ml. of concentrated nitric acid.

† Based on chemical analyses for total serum protein in 24 sheep and 9 goats.[18]

per cent, and a TBW content of 70.3 per cent of body weight, showing wide divergence from the normal. These atypical values could not be rechecked or further studied due to the condition of the animal.**

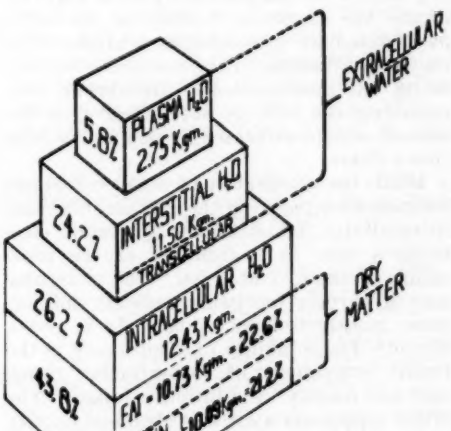
The normal fluid volumes of the several compartments in sheep, goats, and cattle were determined, in addition to abnormal volumes in sheep produced by experimental expansion and contraction of extracellular fluid volume (table 1). Separation of the TBW in a normal and typical sheep into its several component fluid compartments, and the use of this analysis in determining gross body composition, is indicated (table 2). The normal relationship between the various water compartments and their quantitative aspects are illustrated schematically (fig. 1). This serves to facilitate a brief discussion of the concept of an "anatomic" versus a "physiological" volume of the extracellular fluid.

DISCUSSION

The chemistry of the blood has been regarded largely in terms of solute concentration and considered a valid reflection of the volume status of the extracellular fluids. Recent studies of PW volume,[17,35] ECW volume,[19] and TBW [25] in ruminants make it feasible and important to consider body chemistry in terms of actual quantities. Physiological mechanisms operate to maintain normal solute concentrations between the several body fluid compartments, and their volumetric dimensions are either contracted or expanded to maintain this balance. The dietary habits of ruminants, however, impose certain limitations on these physiological mechanisms in the maintenance of normal fluid and electrolyte (Na^+) balance, and aberrations in fluid balance are demonstrated readily by appropriate experimental procedures.[18,19]

Anatomic Versus Physiological Volume of Body Fluids.—The chemical anatomy of the body fluids, as it is currently understood,[10] separates the total body water (TBW) into an extracellular water (ECW) and an intracellular water (ICW) compartment. The extracellular water is divided further into the intravascular or blood plasma water (PW) and the interstitial water (ISW), which fills the spaces surrounding all the various cells and connective tissues of the body. The names applied to these fluid compartments are anatomic and are descriptive of their anatomic sites.

On the basis of the "dilution principle," whereby some substance is introduced into the PW and its dilution volume ascertained, the fluid compartments of the body can be studied. The meaning of the dilution volume of various substances, as determined *in vivo* by dilution analysis (inulin, manitol, sucrose, Na^{24}, Cl^-, Br^-, ^-SCN), has been extended in usage to become synonymous with some specific anatomic space (ECW), whereas the dilution volume signifies only a virtual volume of equilibration. This is indicated by an inulin space of 15 to 16 per cent of the body weight in man and a ^-SCN space of 24 to 26 per cent.[15,31] In the dog, the inulin space equals 19 to 22 per compliments it.[37] However, the ^-SCN space amounts to 29 to 34 per cent in this species, and in both man and the dog the ^-SCN space



Intracellular water=total body water–extracellular water; interstitial water = extracellular water–blood plasma water; fat = 100–percentage total body water/0.726; dry matter = body weight–total body water.

Fig. 1—Schematic illustration of the fluid compartments of the body and their quantitative relation as determined in a sheep. The transcellular component of the extracellular water represents water residing in the comparatively "dead" spaces of the body and the gastrointestinal secretions, and is indicated as an important component of the physiological volume of the extracellular compartment.

** This Hampshire ram, 11 or 12 years old, was extremely lean, as judged from somatotype, and severely debilitated. There was evidence of mild peripheral edema and swelling of the joints.

appears to be consistently 10 per cent greater than the inulin space. It is evident, therefore, that inulin and $^{-}$SCN can not occupy the same anatomic space because of the wide divergence in their apparent volumes of dilution.

On entering the blood stream, the diffusion of a particular substance through single or all fluid phases of the body might in theory be limited by anatomic barriers, chemical barriers, or both. Inulin has a molecular weight of about 5,100, giving this large molecule a low diffusion coefficient,[40] but it is still small enough to allow free exchange between PW and ISW phases. Because inulin is an elongated molecule and because elongation increases the effective radius under conditions of random orientation, it has an effective diffusion coefficient equivalent to a molecular weight of about 15,000.[3] Under these conditions, the distribution of inulin will be principally diffusion limited,[4] and this feature also may free inulin from the possibility of readily entering cells.[36] Consequently, inulin may indicate the anatomic volume of the ECW, as dictated by membranous limitations to its free diffusion. This anatomic barrier, being the semipermeable membrane surrounding the cell, is not effective in the case of $^{-}$SCN, as indicated by its greater dilution volume.

While the penetration of the membranous component separating the extracellular and intracellular compartments by certain electrolytes may be a function of the ionic radius of the hydrated ion,[26] the molecular size of certain inorganic radicals and organic nonelectrolytes apparently is not a factor.[6] The solubility of a substance in the lipoid component of membranous tissue also will modify its dilution volume.[34] The latter represents a distinct chemical barrier to the free diffusion of $^{-}$SCN and, since the firm histological basis for the division of the ECW and ICW compartments presents an anatomic barrier, it is likely that the dilution volume of $^{-}$SCN is conditioned by chemical barriers to its free diffusion. The volume in which a chemical substance, organic (inulin) or inorganic ($^{-}$SCN), becomes diluted in the body fluids can not be ascertained from its empirical formula or physicochemical activity *in vitro*. The *in vivo* measure of the ECW compartment must be contingent, therefore, on a certain degree of empiricism which does not limit its usefulness but does indicate that the anatomic volume (inulin space) and the physiological volume ($^{-}$SCN space) do not coincide, and that their identity is not comparable.

Aside from water in the vascular and interstitial compartments, which comprise the extracellular compartment by anatomic definition, there is water in the gut, saliva, cerebrospinal fluid, and synovial sacs. In most discussions of body water, these important fluid phases are collectively neglected. Thiocyanate is known to distribute itself throughout the ECW compartment, which we refer to as the anatomic volume (inulin space), but, in addition to this space, it enters spinal fluid and gastrointestinal secretions.[5] The volume of distribution of $^{-}$SCN is understandably greater than that of inulin and it measures a more dynamically functional space, the physiological volume. The physiological volume includes, then, the anatomic volume and the transcellular water, or those fluids residing in the comparatively dead spaces and the gastrointestinal secretions. The sum of all extracellular fluids (physiological volume) is now recognized to comprise 20 to 30 per cent of the body weight in man,[7] the dog,[8] and sheep.[19] The $^{-}$SCN space, as indicative of the physiological volume and perhaps synonymous with it, is a more realistic measure of the complete ECW compartment. It is important to recognize this characteristic in the $^{-}$SCN dilution analysis method for, lacking a complete analysis of all ECW, the ICW compartment can not be estimated adequately.

The ECW compartment in ruminants (table 1), as determined by $^{-}$SCN dilution analysis, represents all or a great part of the transcellular water. This transcellular component is, therefore, placed in its rightful place in the interstitial position (fig. 1) and it constitutes about 10 per cent of the $^{-}$SCN space, or physiological volume. By virtue of the large gastrointestinal capacity, characteristic of the ruminant species, and the voluminous salivary flow,[27] the transcellular fraction of the extracellular fluids accordingly may be expected to be greater than that in simple-stomached species. Simultaneously determined inulin and $^{-}$SCN spaces, with their differential volumes of dilution, may afford a valid and useful measure of the transcellular fluid volume.

Body Fluids and Body Composition.—The body fluids, as they are now known to

be related to body composition, can be most useful in studies of energy metabolism (bioenergetics) and in determining production values in nutrition studies in terms of actual quantities of product produced.

A constant relationship between metabolic rate and some body component has long been sought, but without complete success. The finding of a linear relation between logarithm of body weight and logarithm of oxygen consumption led Kleiber [22] to propose the use of the three fourths power of weight as a metabolic reference standard, which has been adopted by the Committee on Animal Nutrition of the National Research Council.[16] Kleiber [22] assumed that body composition does not vary greatly in different animals.

The selection of an accurate metabolic reference standard is particularly important if the influence of differential gross body composition on basal metabolism, and thus nutritional requirement, is to be assessed adequately. As established previously, the mammalian body contains a variable quantity of water and fat (one being inversely proportional to the other), but contains a fat-free lean body mass of constant gross chemical composition. The addition of fat, which is somewhat inert physiologically and thus contributes little to basal metabolic requirements but much to body weight (as much as 40%) and surface area (as much as 13%, should contribute much less to the metabolic picture than the addition of an equal weight of lean body mass or "active tissue mass." Therefore, mathematical abstractions in the form of exponential functions of body weight offer no valid reason for assuming that bioenergetic processes must be geared to surface area. The differential addition of fat to a constant lean body mass of paired animals will, in effect, produce differences in surface area but not a proportional difference in basal metabolic rate. Thus, the normally high fat content of the female body, with subsequently less lean body mass, explains the lower metabolic rate of the female, when compared with the male on the basis of surface area.[21] Lean body mass has been indicated to be the best metabolic reference standard, and body weight and surface area may be useful only as they are correlated with lean body mass.[29]

The mean water content of the lean body mass in the ruminant has been established to be 72.6 per cent.[23] With smaller animals, Pace and Rathbun [33] determined it to be 73.2 per cent, a difference of small consequence. Using the factor, 0.726, the percentage of body fat may be determined from the TBW determination by the formulation of Pace and Rathbun [33] as follows:

$$\text{Percentage of fat} = 100 - \left(\frac{\text{percentage of TBW}}{0.726}\right)$$

The body composition of a wether sheep, calculated from the simultaneously determined ECW and TBW volumes (table 2), consists of approximately 56 per cent water, 23 per cent fat, and 44 per cent dry matter. The lean body mass equals 77 per cent of the gross weight.

A timely and important contribution by Ellenberger *et al.*,[9] revealing the body composition of 132 ruminants as determined by direct chemical analysis, shows good agreement with the case shown (table 2). We have calculated the percentage of body fat from the TBW analyses of Ellenberger *et al.*, and the general agreement is interesting (table 3). The significance of table 3 lies in the fact that the concept of a lean body mass of constant gross chemical composition is supported further, and also that

TABLE 3—Percentage Body Fat of Cattle as Calculated from the Chemical Analyses of Water of Ellenberger et al.[9] *

Chemical analyses of Ellenberger *et al.*[9]				Calculated from percentage of water
Age	No. animals	Water (% body wt.)	Fat (% body wt.)	Fat (% body wt.)
Newborn	7	74.2	2.8	.....
3 mo.	17	71.7	4.8	1.2
6 mo.	16	69.1	7.2	4.9
9 mo.	10	66.2	10.1	8.8
12 mo.	9	63.8	12.7	12.2
2½–3½ yr.	9	58.4	17.9	19.5
3½–4½ yr.	25	59.7	16.4	17.8
4½–5½ yr.	9	58.2	19.0	19.8
6–12 yr.	6	63.0	13.9	13.2
Mean, 9 mo.–12 yr.	68	64.9	15.0	15.5

* The percentage of body fat and water of the bodies of 108 cattle determined chemically by Ellenberger, *et al.*[9] and body fat as calculated from their data on percentage of total body water. Close agreement between body fat determined directly and as calculated from total body water is evident in the 68 animals of 9 months to 12 years of age, thus lending further support of the concept of a lean body mass constant in gross chemical composition. The data (calculated fat) also indicate that "chemical maturity" may not be attained in the bovine species until about 9 months of age.

XUM

this concept may not be valid in cattle younger than 9 months of age.

Physiological or "chemical" maturity has been recognized to vary among mammals, with man requiring the longest period for its attainment. Moulton[30] has indicated that chemical maturity in cattle is acquired at about 5 months of age. If chemical maturity can be defined as that age when the gross chemical composition of the lean body mass becomes constant or the water content of the lean body mass stabilizes at 73 per cent, then the data of Ellenberger *et al.*[9] (table 3) indicate that chemical maturity occurs in cattle at about 9 months of age. It is important to recognize chemical maturity as a limiting factor in the use of TBW analysis in the indirect determination of body composition.

Changes in volume of the fluid compartments under conditions of hydration and dehydration in the sheep (table 1) emphasize the importance of the simultaneous determination of ECW and TBW. Severe dehydration, as produced by low dietary sodium intake,[19] in 2 sheep (194 and 228) reduced the PW 26 per cent and the ISW and ECW 25 per cent of their normal values. A TBW content of 50 and 48 per cent for these animals, indicating 31 and 33 per cent body fat, respectively, is grossly in error as judged from somatotype. The dehydration is generalized and not confined to the ECW compartment. The decrease in PW and ISW is equal; thus, the plasma volume is not maintained in response to unavoidable loss of electrolyte (Na^+) in the endeavor to maintain its normal solute concentration.[18,19]

For general application in studies of composition, and especially in studies of fluid balance, the TBW determination should be accompanied by the simultaneously determined ECW volume to avoid gross errors arising from differences in hydration. The general usefulness, simplicity, and accuracy in quantitative measures of the fluid components of the body warrant further study and application.

SUMMARY

A procedure for the simultaneous determination of the extracellular and total body water using sodium thiocyanate and antipyrine was described.

Normal extracellular water volume (thiocyanate space) in sheep and goats is consistent at 30.0 per cent of body weight; cattle 8 to 9 months old show 28.0 per cent. Normal plasma water volume in 18 wethers and 9 goats, as determined by dye (T-1824) dilution analysis, averaged 5.8 per cent of body weight. Blood plasma thiocyanate concentrations in sheep and goats decreased exponentially at the rate of 4.0 to 5.0 per cent of the injected dose per hour, and equilibrium with the extracellular water was reached 60 to 90 minutes postinjection. Cattle showed a consistent plasma concentration of thiocyanate for six hours postinjection. Antipyrine was metabolized by the sheep and goat at the rate of 28.0 to 30.0 per cent per hour of the injected dose, while in cattle its metabolism was slower by about 5.0 per cent.

The body composition of a wether is given as determined from the analysis *in vivo* of the several fluid compartments.

Severe dehydration in the sheep produced by low dietary sodium intake resulted in a 26 per cent reduction in plasma water volume, and a 25 per cent reduction in interstitial and extracellular water volume.

The concept of an anatomic versus a physiological volume of the extracellular water compartment is introduced, the latter being synonymous with the thiocyanate space.

References

[1] Berger, E. Y., Dunning, M. F., Steele, J. M., Jackenthal, R., and Brodie, B. B.: Estimation of Intracellular Water in Man. Am. J. Physiol., *162*, (1950): 318–325.

[2] Brodie, B. B., Axelrod, J., Soberman, R., and Levy, B. B.: The Estimation of Antipyrine in Biological Materials. J. Biol. Chem., *179*, (1949): 25–29.

[3] Bunim, J. J., Smith, W. W., and Smith, H. W.: The Diffusion Coefficient of Inulin and Other Substances of Interest in Renal Physiology. J. Biol. Chem., *118*, (1937): 667–681.

[4] Cotlove, Earnest: Mechanism and Extent of Distribution of Inulin and Sucrose in Chloride Space of Tissues. Am. J. Physiol., *176*, (1954): 396–410.

[5] Crandall, Lathan A., and Anderson, Milford X.: Estimation of the State of Hydration of the Body by the Amount of Water Available for the Solution of Sodium Thiocyanate. Am. J. Dig. Dis., *1*, (1935): 126–131.

[6] Davson, H., Duke-Elder, S., Maurice, D. M., Ross, E. J., and Woodin, A. M.: The Penetratior of Some Electrolytes and Non-Electrolytes into the Aqueous Humor and Vitreous Body of the Cat. J. Physiol. (London), *108*, (1949): 203–217.

[7] Edelman, I. S., Olney, J. M., James, A. H., Brooks, L., and Moore, F. D.: Body Composition: Studies in the Human Being by the Dilution Principle. Science, *115*, (1952): 447–454.

[8] Elkinton, J. R., and Taffel, M.: The Apparent Volume of Distribution of Sulfocyanate and of Sulfanilamide in the Dog. Am. J. Physiol., *138*, (1942): 126–135.

[9] Ellenberger, H. B., Newlander, J. A., and Jones, C. H.: Composition of the Bodies of Dairy Cattle. Vermont Agric. Exper. Sta. Bull. 558, (1950): 1–66.

XUM

[10] Gamble, James L.: Extracellular Fluid. 5th ed. Harvard University Press, Cambridge, Mass., 1951.

[11] Goldstein, Franz, and Rieders, Fredric: Conversion of Thiocyanate to Cyanide by an Erythrocytic Enzyme. Am. J. Physiol., *173*, (1953): 287–290.

[12] Gorman, W. F., Messinger, E., and Herman, M.: Toxicity of Thiocyanates Used in Treatment of Hypertension. Ann. Intern. Med., *30*, (1949): 1054–1056.

[13] Gregerson, Magnus I.: Blood Volume. Ann. Rev. Physiol., *13*, (1951): 397–412.

[14] Guadino, M., and Levitt, M. F.: Inulin Space as a Measure of Extracellular Fluid. Am. J. Physiol., *157*, (1949): 387–393.

[15] Guadino, M. Schwartz, I. L., and Levitt, M. F.: Inulin Volume of Distributiou as a Measure of Extracellular Fluid in Dog and Man. Proc. Soc. Exptl. Biol. & Med., *68*, (1948): 507–511.

[16] Guilbert, H. R., and Loosli, J. K.: Comparative Nutrition of Farm Animals. J. Anim. Sci., *10*, (1951): 22–31.

[17] Hansard, Sam L., Butler, William O., Comar, C. L., and Hobbs, C. S.: Blood Volume of Farm Animals. J. Anim. Sci., *12*, (1953): 402–406.

[18] Hix, Elliott L.: The Physiological Significance of Sodium, Potassium and Chloride as Related to the Body Fluids and the Von Bunge Theory of Sodium-Potassium Antagonism. Ph.D. thesis, Kansas State College, Manhattan, 1953.

[19] Hix, Elliott L., Evans, L. E., and Underbjerg, G. K. L.: Extracellular Water and Dehydration in Sheep. J. Anim. Sci., *12*,, (1953): 459–473.

[20] Kennedy, J. A., and Millikan, G. A.: A Micro Blood Volume Method Using a Blue Dye and Photocell. J. Physiol. (London), *93*, (1938): 276–284.

[21] Keys, Ancel, and Brozek, Josef: Body Fat in Adult Man. Physiol. Rev., *33*, (1953): 245–325.

[22] Kleiber, Max: Body Size and Metabolic Rate. Physiol. Rev., *27*, (1947): 511–541.

[23] Kraybill, H. F., Bitter, H. L., and Hankins, O. G.: Body Composition of Cattle. II. Determination of Fat and Water Content from Measurement of Body Specific Gravity. J. Appl. Physiol., *4*, (1952): 575–583.

[24] Kraybill, H. F., Goode, E. R., Robertson, S. B., and Sloane, H. S.: *In Vivo* Measurement of Body Fat and Body Water in Swine. J. Appl. Physiol., *6*, (1953): 27–32.

[25] Kraybill, H. F., Hankins, O. G., and Bitter, H. L.: Body Composition of Cattle. I. Estimation of Body Fat from Measurement *in Vivo* of Body Water by Use of Antipyrine. J. Appl. Physiol., *3*, (1951): 681–689.

[26] Ling, G.: Selective Ionic Accumulation in Muscle Cell. Fed. Proc., *11*, (1952): 95.

[27] McDougall, E. I.: Studies of Ruminant Saliva: The Composition and Output of Sheep's Saliva. Biochem. J., *43*, (1948): 99–106.

[28] Miller, A. T., and Blyth, Carl S.: Estimation of Lean Body Mass and Body Fat from Basal Oxygen Consumption and Creatinine Excretion. J. Appl. Physiol., *5*, (1952): 73–78.

[29] Miller, A. T., Jr., and Blyth, Carl S.: Lean Body Mass as a Metabolic Reference Standard. J. Appl. Physiol., *5*, (1953): 311–316.

[30] Moulton, C. R.: Age and Chemical Development in Mammals. J. Biol. Chem., *57*, (1923): 79–92.

[31] Odier, Jacques: La measure du volume du liquide extracellulaire chez l'homme au moyen de l'epreuve au sulfocyanure de sodium. Helv. med. Acta., *15*, (1948): 1–56.

[32] Pace, Nello, Kline, Leo, Schachman, Howard K., and Harfenist, Morton: Studies on Body Composition. IV. Use of Radioactive Hydrogen for Measurement *in Vivo* of Total Body Water. J. Biol. Chem., *168*, (1947): 459–469.

[33] Pace, Nello, and Rathbun, Edith N.: Studies of Body Composition. III. The Body Water and Chemically Combined Nitrogen Content in Relation to Fat Content. J. Biol. Chem., *158*, (1945): 685–691.

[34] Pappenheimer, John R.: Passage of Molecules Through Capillary Walls. Physiol. Rev., *33*, (1953): 387–423.

[35] Reynolds, Monica: Plasma and Blood Volume in the Cow Using the T-1824 Hematocrit Method. Am. J. Physiol., *173*, (1953): 421–427.

[36] Robinson, J. R., and McCance, R. A.: Water Metabolism. Ann. Rev. Physiol., *14*, (1952): 115–142.

[37] Sapirstein, L. A., Herrold, M. R., Janakis, M., and Ogden, E.: Validity of Values for Glomerular Filtration Rate and Extracellular Fluid Obtained from Plasma Concentration-Time Decay Curves After Single Injections of Mannitol in the Dog. Am. J. Physiol., *171*, (1952): 487–491.

[38] Soberman, R., Brodie, B. B., Levy, B. B., Axelrod, J., Hollander, V., and Steele, J. M.: The Use of Antipyrine in the Measurement of Total Body Water in Man. J. Biol. Chem., *179*, (1949): 31–42.

[39] Steele, J. M., Berger, E. Y., Dunning, M. F., and Brodie, B. B.: Total Body Water in Man. Am. J. Physiol., *162*, (1950): 313–317.

[40] Westfall, B. B., and Landis, E. M.: The Molecular Weight of Inulin. J. Biol. Chem., *116*, (1936): 727–730.

XUM

Comparison of Bacteriological and Serological Techniques for Detection of Leptospirosis in Wild Mammals

STURGIS McKEEVER, Ph.D.; JOSEPH H. SCHUBERT, Ph.D.;
GEORGE W. GORMAN, B.S.; ROGER D. GRIMES, B.S.

Atlanta and Newton, Georgia

SEROLOGICAL TESTS have been used extensively as a method for determining whether an animal is, or has been, infected with leptospiras, and such tests are considered to be the most useful laboratory tool for confirmation of the diagnosis of leptospirosis.[8] The complement-fixation and agglutination-lysis tests are the two most commonly used. Yager and Gochenour [15] found the two tests equally reliable and specific when compared to a series of bacteriologically proved cases. For single serum examinations, they considered an agglutination-lysis titer of 1:400 or greater, or a 4+ complement-fixation reaction with undiluted serum, to be of diagnostic significance. The complement-fixation test is less tedious to perform than the agglutination-lysis test,[12] and complement-fixing antigens react to a broader spectrum of serotypes than those employed in the agglutination-lysis test.[2]

Gochenour [4] states that, on the average, agglutinating antibodies are detectable on the twelfth day and reach their highest level the second and third week after infection; they may be demonstrated as early as the seventh day and persist for as long as 500 days. Complement-fixing antibodies may be detected as early as the second day after infection but, on the average, tests become positive on the tenth day; they persist for varying lengths of time, but in human beings and cattle they generally can not be detected after the third month.

Leptospiras may be detected bacteriologically by culturing blood, cerebrospinal fluid, anterior chamber fluid, urine, and tissue emulsions.[4] In cattle, leptospiras are present in the blood as early as one day after parenteral inoculation and may be recovered from the blood during the prefebrile and febrile period. They become established in the kidney during the febrile period and may then be shed in the urine for six to eight weeks.[9]

At Newton, Ga., the U.S. Public Health Service has conducted investigations on the role of wildlife in the propagation of leptospiras and has used both complement-fixation and bacteriological tests for detecting infection. This paper presents a comparison of the results obtained by the two methods from Oct. 4, 1955, to April 19, 1957.

MATERIALS AND METHODS

Wild mammals were trapped and brought to the laboratory alive. Blood samples were obtained by cardiac puncture, and the animals were killed by injecting air into the blood stream. The kidneys of each animal were removed aseptically and ground in sufficient saline solution, adjusted to a pH of 7.2 to 7.4 with sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$), to make a 10 to 15 per cent tissue suspension. Two drops of the suspension was used to inoculate each of four screw-capped culture tubes containing 5 ml. of medium. The following mediums were used: Chang's [1] semisolid medium with 10 per cent rabbit plasma, Chang's semisolid medium with 15 per cent horse plasma, Fletcher's [2] semisolid medium with 10 per cent rabbit plasma, and Fletcher's semisolid medium with 15 per cent horse plasma. The Hamilton, Mont.[10] modification of Chang's medium was used. After inoculation, the tubes of mediums were incubated for 30 days at 28 C. and then examined for leptospiras by dark-field procedure.

Strains of leptospiras obtained from these cultures were typed by the Leptospira Research Laboratory of the Communicable Disease Center, Laboratory Branch.

Cells were removed from the blood by centrifugation, and the serums were frozen and shipped weekly to the Diagnostic Serology Unit of the Communicable Disease Center, Laboratory Branch, to be tested for the presence of complement-fixing antibodies.

The serums were inactivated at 56 C. for 30 minutes and tested in serial dilutions, beginning with a dilution of 1:4, with antigens of *Leptospira ballum, Leptospira pomona, Leptospira icterohaemorrhagiae,* and *Leptospira canicola.* Cultures for antigens were grown on a modified Schuffner's

From the Communicable Disease Center, Bureau of State Services, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Ga.; and Emory University Field Station, Newton, Ga.

medium [11] and were prepared as reported by Schubert and Martin.[12]

The complement-fixation procedure used was an adaptation of the 50 per cent end point technique, using barbital buffered physiological saline solution throughout. Antigens and serial dilutions of inactivated serums were tested in 0.25 ml. amounts with 0.25 ml. of complement containing three and one half 50 per cent units. Fixation was allowed to proceed for 15 to 18 hours at 4 to 6 C. Sensitized cells (a 2% suspension of sheep erythrocytes, sensitized for ten minutes in the proportion of 1:1 with three 50% units of hemolysin in 0.125 ml.) were added in 0.25 ml. amounts, and the tests were read after a second incubation period of 30 minutes in a 37-C. water bath. The titers were recorded as the highest dilution of serum which gave a 3+ or 4+ reaction (30% hemolysis or less).

RESULTS

During the period of study, 2,501 animals, representing 14 species, were trapped. Of these, 1,811 were tested for the presence of leptospiras by culturing kidney tissue, and 181 (10%) were positive; complement-fixation tests were performed on 2,200, and 399 (18%) were positive.

Both culture and complement-fixation tests were performed on 1,704 animals (table 1). Of these, 162 (9.5%) were positive by culture and 307 (18.0%) were positive by complement-fixation tests. For the seven species which had 1 or more individuals that were positive by both tests, the incidence of infection was 0.5 to 28.6 per cent by culture and 6.9 to 42.1 per cent by complement-fixation tests. Only 60 (37%) of the animals from which isolates were obtained were positive by the complement-fixation test; agreement with culture isolations ranged from 11 to 100 per cent for the seven species represented. Titers and serotypes of isolates obtained from animals that were positive by both tests are presented (table 2). Titers ranged from 1:4 to 1:1,024; for every identified isolate where the host had a positive complement-fixation test, the highest or an equal titer was obtained with antigens of the same serotype as the isolate.

The number of animals that were positive by the complement-fixation test equalled or exceeded the number that were positive by culture for all species except the opossum, in which more than twice as many were positive by culture. Complement-fixation tests were performed on 630 opossums but, with one exception, reactions were obtained only with antigens of *L. ballum*. Reactions of the serums to the four antigens against which they were

TABLE 1—Comparison of Results of Bacteriological and Complement-Fixation (CF) Tests for Leptospirosis in Mammals from Southwestern Georgia

Species	No. of duplicate tests	No. of positive cultures	No. of positive CF tests	No. of positive culture and CF tests	Positive by culture (%)	Positive by CF test (%)	Positive by culture and CF test (%)
Opossum *Didelphis marsupialis*	464	78	32	23	16.8	6.9	29.5
Cottontail rabbit *Sylvilagus floridanus*	187	1	58	1	0.5	31.0	100.0
Marsh rabbit *Sylvilagus palustris*	1	....	1	....	....	100.0	.....
Gray squirrel *Sciurus carolinensis*	2	....	....	....	....	.....	.....
Fox squirrel *Sciurus niger*	41	....	....	....	....	.....	.....
Red fox *Vulpes fulva*	16	....	4	....	....	25.0	.....
Gray fox *Urocyon cinereoargenteus*	107	5	45	2	4.7	42.1	40.0
Dog *Canis familiaris*	1	....	1	....	....	100.0	.....
Raccoon *Procyon lotor*	506	36	46	4	7.1	9.1	11.1
Striped skunk *Mephitis mephitis*	276	36	100	26	13.0	36.2	72.2
Spotted skunk *Spilogale putorius*	7	2	2	1	28.6	28.6	50.0
Otter *Lutra canadensis*	2	....	1	....	....	50.0	.....
Wildcat *Felis rufa*	62	4	15	3	6.5	24.2	75.0
House cat *Felis domestica*	32	....	2	....	....	6.2	.....
Total	1,704	162	307	60	9.5	18.0	36.4

XUM

TABLE 2—Range of Complement-Fixation End Point Titers for Serums from Mammals Bacteriologically Confirmed as Infected with Leptospiras

Species	No. of isolates	Serotype of isolates	Antigen			
			L. ballum	*L. pomona*	*L. icterohaemorrhagiae*	*L. canicola*
Striped skunk	1	*L. ballum*	1:16	.....	.....	.....
	7	*L. pomona*	.....	1:8–1:256	1:16(1)*	.....
	18	Unidentified	1:4–1:64(14)	1:4–1:1,024(13)	1:4–1:64(10)	1:4–1:256(7)
Opossum	1	*L. ballum*	1:16	.....	.....	.....
	22	Unidentified	1:4–1:64(22)	.....	.....	.....
Wildcat	2	*L. pomona*	.....	1:64	1:8(2)	1:8(1)
	1	Unidentified	1:16	1:8	.....	1:64
Cottontail rabbit	1	Unidentified	1:8	1:8	1:4	1:4
Gray fox	2	Unidentified	1:16–1:32	1:4–1:32	1:4–1:16	1:4–1:16
Raccoon	4	Unidentified	1:4–1:16(2)	1:4–1:8(2)	1:8(1)	1:32(1)
Spotted skunk	1	Unidentified	1:8	.....	.....	.....

* Number shown in parentheses indicates the number of animals which reacted to this species of Leptospira.

tested are presented by species (table 3). The serums of many animals had titers to antigens of more than one serotype and are tabulated.

Of the 181 isolates, 44 have been identified. Distribution by hosts and results of complement-fixation tests on the 39 of these animals that were so tested are presented (table 4).

DISCUSSION

The complement-fixation and agglutination-lysis tests have been used both in diagnosing leptospirosis in man and animals and in surveys designed to determine the presence or absence of leptospirosis in large numbers of domestic animals. Both tests are relatively inexpensive and can be performed in a short time. Theoretically, in either test a combination of antigens can be used which will detect the presence of antibodies of any serotype of Leptospira. Paired serums, in which the second serum of the pair shows an increase in titer for the antigen of one serotype, are required to determine the strain of the organism involved. Although the validity of these tests for the detection of leptospiral antibodies in man and domestic animals has been confirmed, their accuracy for detecting infections of leptospiras in wild mammals is open to question. Results of complement-fixation tests of serums from wild mammals are discussed in this paper. The merits of the agglutination-lysis test under similar conditions are unknown.

When the bacteriological test is used, false positives are eliminated and organisms are recovered which can be identified accurately as to serotype, or characterized if not previously described. Leptospiras may be detected in the prefebrile period, when they would be missed by serological tests, but may not be detected in the late postfebrile period. All stages of the bacteriological test must be conducted with exact-

TABLE 3—Range of End Point Titers for Serums of Mammals from Southwestern Georgia in Complement-Fixation Tests with Antigens of Four Serotypes of Leptospiras

Species	No. tested	Antigen							
		L. ballum		*L. pomona*		*L. icterohaemorrhagiae*		*L. canicola*	
		No. positive	Titer	No. positive	Titer	No. positive	Titer	No. positive	Titer
Opossum	630	34	1:4–1:256	....	.....	.	.....	1	1:8
Cottontail rabbit	203	35	1:4–1:128	42	1:4–1:256	49	1:4–1:1,024	42	1:4–1:1,024
Marsh rabbit	4	1	1:16	2	1:16–1:64	2	1:16	1	1:16
Gray squirrel	2	....	.....	....	.....	....	.....	....	.....
Fox squirrel	47	....	.....	1	1:64	....	.....	....	.....
Red fox	24	4	1:4–1:64	5	1:8–1:2,048	6	1:4–1:128	5	1:4–1:512
Gray fox	145	39	1:4–1:128	49	1:4–1:256	28	1:4–1:128	32	1:4–1:64
Dog	1	....	.....	1	1:32	1	1:32	1	1:16
Raccoon	640	8	1:4–1:64	37	1:4–1:256	23	1:4–1:64	17	1:4–1:32
Striped skunk	362	87	1:4–1:2,048	85	1:4–1:1,024	60	1:4–1:256	40	1:4–1:256
Spotted skunk	10	2	1:4–1:8	1	1:4	....	.....	....	.....
Otter	2	....	.....	....	.....	1	1:32	1	1:8
Wildcat	94	14	1:8–1:64	18	1:4–1:512	7	1:4–1:32	8	1:4–1:64
House cat	36	1	1:8	1	1:8	....	.....	....	.....

ness, or the leptospiras will die and the result will be a false negative test. Since the bacteriological test is more tedious, requires more equipment, personnel, and time than the serological test, it is more a tool of the research worker than of the diagnostician or those conducting surveys.

Disagreement between bacteriological and serological tests for leptospirosis may be attributed to one or more of the following five factors: (1) testing the animal after infection but prior to antibody formation; (2) testing after the disease has terminated but with antibodies persisting; (3) insufficient reaction between antigens being used and antibodies of other serotypes of leptospiras; (4) antigen-antibody reaction not being the same for all species of hosts; and (5) missing the organism by bacteriological tests, even though it is present.

After an antigen is introduced into the body, there is a period during which no antibodies can be detected, followed by a rapid rise in antibody level until a peak is reached. The peak is followed by a rapid decrease and then a gradual decline in antibody level over a relatively long period.[14] It follows that leptospiral infections which are detectable by culture should also be detectable serologically, except for the period prior to antibody formation.

The time of initial detection and the persistence of complement-fixing antibodies may vary for different serotypes of leptospiras as well as for different hosts infected with a given serotype of Leptospira. Limited results from animals experimentally infected with *L. pomona* indicate that gray and red foxes have antibodies at the end of one week which persist for at least eight weeks; raccoons have antibodies at the end of two weeks which are detectable for at least seven weeks; and opossums, wildcats, and striped skunks have antibodies at the end of two weeks (perhaps a week earlier) which persist for at last three, five, and seven weeks, respectively. The period required for complete loss of detectable antibodies has not been determined for any of these species.

Isolates of leptospiras were obtained from the cottontail rabbit and spotted skunk, which have not been reported previously as hosts, but no experimental studies were made with these species. Identification of isolates obtained from these hosts has not been completed.

From the foregoing results, and from data reported by Gochenour [4] for other species, one would expect more than 60 per cent agreement between bacteriological and serological tests. However, of the 24 infections determined to be either *L. ballum* or *L. pomona,* only 11 (46%) were detected by the complement-fixation test (table 4). These 11 showed serotype agreement by the two tests. It is also pertinent that of the 15 isolates of serogroups not used in the complement-fixation tests, none were detected by such tests.

The present study has already demonstrated that opossums are infected with organisms of the *Leptospira hyos* serogroup, and raccoons with *Leptospira australis* A and organisms of the *Leptospira grippoty-*

TABLE 4—Results of Complement-Fixation Tests of Serums from Mammals Infected with Known Types of Leptospiras

Species	Serogroup	No. of isolates	Positive		Negative*
			L. ballum	*L. pomona*	
Opossum	*L. ballum*	4	1	..	3
	L. hyos†	3(1)	..	..	3
Gray fox	*L. ballum*	2	..	..	2
Raccoon	*L. ballum*	1	..	..	1
	L. pomona	(1)	..	..	..
	L. australis A†	2	..	..	2
	L. grippotyphosa†	2	..	..	2
	L. autumnalis†	5(2)	..	..	5
	L. hebdomadis†	2	..	..	2
	L. hyos†	1	..	..	1
Striped skunk	*L. ballum*	5	1	..	4
	L. pomona	9(1)	..	7	2
Wildcat	*L. ballum*	1	..	..	1
	L. pomona	2	..	2	0
Totals		39(5)**	2	9	28

**Leptospira icterohaemorrhagiae* and *L. canicola* antigens were used also but all were negative. † Homologous antigens not used in the complement-fixation test. ** Complement-fixation tests were not made on hosts of five identified isolates shown in parentheses.

XUM

phosa, Leptospira autumnalis, Leptospira hebdomadis, and *L. hyos* serogroups. Organisms which were isolated from soldiers with Fort Bragg fever, in 1944, were later identified by Gochenour *et al.*[5] as *L. autumnalis,* but the other three organisms have not been isolated previously in the United States.[3, 6, 7] Serums from animals infected with these organisms gave no reaction with the antigens used. A marked reduction in percentage of agreement between the two tests would, therefore, be expected for the opossum and raccoon, and this is confirmed by the results (table 1).

Schubert *et al.*[12] demonstrated that supernatant antigens failed to give a positive reaction with serums from naturally infected human beings and cattle, whereas whole Leptospira antigens gave good complement-fixation titers with serums from man, cattle, and dogs. Supernatant antigens were used exclusively in the present study and may not have reacted with serums from some species unless the antibody level was near the peak.

Since antibodies persist for some time after the organism is destroyed, the number of animals that are positive by the complement-fixation test should exceed the number that are positive by culture. This was found to be true for all species except the opossum. However, if leptospiras are not isolated from an animal when they are present and serum from the animal is positive, the percentage of agreement between the two tests would be further reduced. It is necessary, therefore, that the best bacteriological test available be used.

Blood frequently is cultured to confirm a diagnosis of leptospirosis in man or domestic animals, as well as in studies with experimentally infected animals. Gochenour[4] found heart blood cultures positive more frequently than tissue cultures, but 85 per cent of the blood culture isolates were made from specimens obtained in the third to eighth day after infection. Since leptospiras can be isolated from the blood during the prefebrile and febrile period only, blood cultures would miss organisms present in kidney tissue over the relatively long postfebrile period.

The culture of urine has received some attention in the diagnosis of leptospirosis. For recovery of leptospiras from man, Yager and Gochenour[15] recommended intraperitoneal inoculation of laboratory animals with urine, followed by blood cultures on the fourth and sixth days postinoculation. Gochenour[4] found inoculation of laboratory animals far less effective than direct cultures for demonstrating the presence of leptospiras in human blood. Presumably, the same would be true for urine, but direct culture of urine is feasible only when it can be removed from the bladder aseptically. Furthermore, the direct culture of urine is of questionable value for determining the incidence of infection in a population composed of many species and infected with several serotypes of leptospiras. The writers have tested both tissue and urine from 35 wild mammals and found 14 positive by culture of tissue only and 1 positive by culture of both tissue and urine.

Although the culture of kidney tissue is feasible only when the animal can be killed, in view of the foregoing it is considered superior to the culture of either blood or urine for detecting leptospiras in wild mammals. Other tissues were not cultured, so results are not available for comparison with cultures of kidney tissue.

SUMMARY

In a study of leptospirosis in wild mammals in southwestern Georgia, 2,501 animals were collected. Of these 1,811 were tested for the presence of leptospiras by culturing kidney tissue; 181 (10%) were positive. Complement-fixation tests using antigens of *Leptospira ballum, Leptospira pomona, Leptospira icterohaemorrhagiae,* and *Leptospira canicola* were performed on 2,200 animals; 399 (18%) were positive.

The tests were duplicated on 1,704 animals; 162 (9.5%) were positive by culture and 307 (18.0%) were positive by complement-fixation tests. Only 60 (37%) of the animals from which isolates were obtained were positive by complement-fixation tests. Agreement of results obtained from the two tests ranged from 11 to 100 per cent for the seven species found positive by both methods.

The absence of cross reaction between the antigens used and antibodies of *Leptospira australis* A and the *Leptospira hyos, Leptospira grippotyphosa, Leptospira autumnalis,* and *Leptospira hebdomadis* serogroups probably caused the greatest discrepancy between the two tests. Prior to the present study, *L. autumnalis* was isolated from

human beings, but a natural host was unknown in North America; *L. australis* A and organisms of the *L. hyos, L. grippotyphosa,* and *L. hebdomadis* serogroups had not been isolated previously in the United States.

Of the 44 isolates identified, 26 were either *L. ballum* or *L. pomona;* blood from 24 of these infected animals was tested, and only 11 (46%) had a positive complement-fixation reaction. Better correlation between the two tests for animals infected with these two serotypes might be expected.

The cottontail rabbit and spotted skunk are recorded as new hosts for leptospiras.

References

[1] Chang, S. L.: Studies on *Leptospira Icterohemorrhagiae.* J. Infect. Dis., *81,* (1947): 28–34.

[2] Fletcher, W.: Leptospirosis, Tsutsugamuski Disease and Tropical Typhus in the Federated Malay States. Tr. Roy. Soc. Trop. Med. & Hyg., *21,* (1927–1928): 265–282.

[3] Galton, M. M., Powers, D. K., McKeever, S., and Gorman, G. W.: Identification of Two Leptospiral Serotypes New to the United States. Pub. Health Rep., *72,* (1957): 431–435.

[4] Gochenour, W. S., Jr.: The Laboratory Diagnosis of Leptospirosis. In "Symposium on the Leptospiroses." Army Medical Services Graduate School, Walter Reed Army Medical Center, Medical Science Publication No. 1 (1953): 160–165.

[5] Gochenour, W. S., Jr., Smadel, J. E., Jackson, E. B., Evans, L. B., and Yager, R. H.: Leptospiral Etiology of Fort Bragg Fever. Pub. Health Rep., *67,* (1952): 811–813.

[6] McKeever, S., Gorman, G. W., Chapman, J. F., Galton, M. M., and Powers, D. K.: Incidence of Leptospirosis in Wild Mammals from Southwestern Georgia, with a Report of New Hosts for Six Serotypes of Leptospirae. Am. J. Trop. Med. & Hyg. In press.

[7] McKeever, S., Gorman, G. W., Galton, M. M., and Hall, A. D.: The Raccoon, *Procyon Lotor,* a Natural Host of *Leptospira Autumnalis.* Am. J. Hyg., *66,* (1958): 13–14.

[8] Reinhard, K. R.: Present Knowledge and Concepts of Leptospirosis in Farm Animals. J.A.V.M.A., *123,* (Dec., 1953): 487–493.

[9] Reinhard, K. R.: Bovine Leptospirosis. In "Symposium on the Leptospiroses." Army Medical Services Graduate School, Walter Reed Army Medical Center, Medical Science Publication No. 1 (1953): 126–139.

[10] Reinhard, K. R.: Leptospiral Techniques. Mimeographed report, Rocky Mountain Laboratory, U.S. Public Health Service, 1953.

[11] Schubert, J. H.: A Simplified Procedure for the Preparation of Schuffner's Leptospira Medium. Pub. Health Lab., *13,* (1955): 67–70.

[12] Schubert, J. H., Carrington, L. B., Conner, E., and Holderman, L. V.: Whole Leptospira Suspensions as Antigens in the Complement-Fixation Test for Leptospirosis. Am. J. Hyg., *66,* (1956): 254–260.

[13] Schubert, J. H., and Martin, D. S.: The Evaluation of Serologic Tests for Leptospirosis. J. Lab. & Clin. Med., *48,* (1956): 155–156.

[14] Smith, D. T., and Martin, D. S.: Zinsser's Textbook of Bacteriology. Appleton-Century-Crofts, Inc., New York, N.Y. 1948.

[15] Yager, R. H., and Gochenour, W. S., Jr.: Leptospirosis in North America. Am. J. Trop. Med. & Hyg., *1,* (1952): 457–461.

XUM

Follicular Acariasis of Mice Caused by *Psorergates Simplex* Successfully Treated with Aramite

ROBERT J. FLYNN, D.V.M.

Lemont, Illinois

INFESTATION of laboratory mice with the follicle-inhabiting mite *Psorergates simplex*[2] is characterized by the formation of skin pouches (fig. 1). The development of a pouch apparently is initiated when one or more mites gain entry into a hair follicle, presumably by way of the pilosebaceous orifice. The result appears as an invagination of the skin that is lined with epidermis, filled with mites and debris, and possesses an opening to the outside (fig. 2, 3). A typical pouch contains several generations of mites in all stages of development (fig. 4).

In an earlier series of experiments directed toward controlling nonfollicular acariasis of mice,[1] numerous acaricides were tested, *i.e.*, rotenone, piperonyl butoxide, pyrethrins, pine oil, DMC [1,1-bis-(p-chlorophenyl) ethanol], BHC (γ-benzene hexachloride), and aramite [2-(p-tert-butylphenoxy) isopropyl-2-chloroethyl sulfite]. Aramite was found to be the most effective and least toxic in the treatment of nonfollicular acariasis. Its effectiveness for the treatment of follicular acariasis caused by *P. simplex* is described in the present study.

MATERIALS AND METHODS

Female CF No. 1 mice, 9 to 13 months old, were randomly distributed, 10 per plastic cage, and bedded with white pine shavings. The cages and bedding were changed twice a week.

A 2 per cent aqueous suspension of a wettable powder containing 15 per cent aramite was used. To insure maximum penetration of the acaricide, a wetting agent, sodium alkylarylsulfonate, was added to the suspension in the amount of 1 Gm. per liter.

Fig. 1—Inner surface of mouse skin showing macroscopic pouches (arrow).

The mice to be treated were placed in a perforated container in groups of ten, completely submerged in the acaricidal suspension for one second, forced to swim for 15 seconds, and submerged again for one second. They were then placed in clean cages.

From the Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Ill. This work was performed under the auspices of the U.S. Atomic Energy Commission.

Presented at the Second Annual Meeting of the American Board of Laboratory Animal Medicine, Aug. 17, 1958, Philadelphia, Pa.

The author thanks Mr. A. S. Tracy for his photographic assistance.

TABLE 1—Effects of Treatment with Aramite on Psorergates Simplex Infestation

No. treatments (weekly interval)	Total No. mice treated	No. mice found to be infested	Total No. pouches found	No. pouches containing live mites	Pouches containing live mites (%)
0	60	32	149	114	76.5
1	60	31	143	18	12.6
2	60	36	143	5	3.5
3	60	36	153	0	0.0

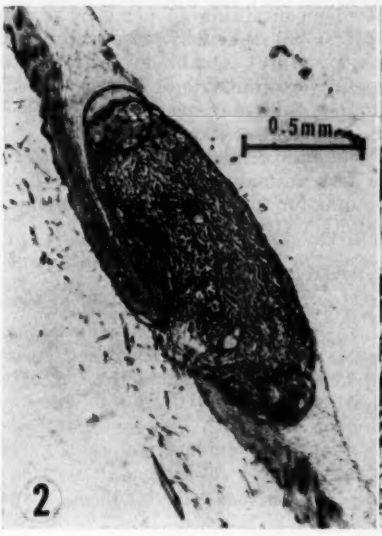


Fig. 2—Section of a typical pouch as shown in figure 1.
Fig. 3—Magnification of circled area of figure 2 (notice mites, arrows).

In all, four groups of 60 mice each were treated as follows: The first group was not treated; the second group was treated once; the third group was treated twice (one-week interval); and the fourth group was treated three times (one-week intervals).

The mice were killed by cervical fracture seven to 14 days after the last treatment. The skin was retracted, the inner surface was examined, and all observable macroscopic pouches produced by *P. simplex* were counted. The contents of these pouches were teased out, suspended in paraffin oil, and immediately examined microscopically for the presence of live mites.

RESULTS

The results are summarized (table 1). In the untreated group of mice, 76.5 per cent of the skin pouches were found to contain live mites. Only 12.6 per cent of the skin pouches contained live mites in mice treated once with aramite, and 3.5 per cent in mice treated twice. Following three weekly treatments with the compound, none of 153 skin pouches examined contained live mites.

DISCUSSION

The results obtained in this study indicate that an aqueous suspension of aramite, combined with a wetting agent, is capable of penetrating the infested follicles and killing the follicle-inhabiting mite, *P. simplex*.

The results also suggest the use of mice infested with *P. simplex* as test animals to screen compounds for topical as well as

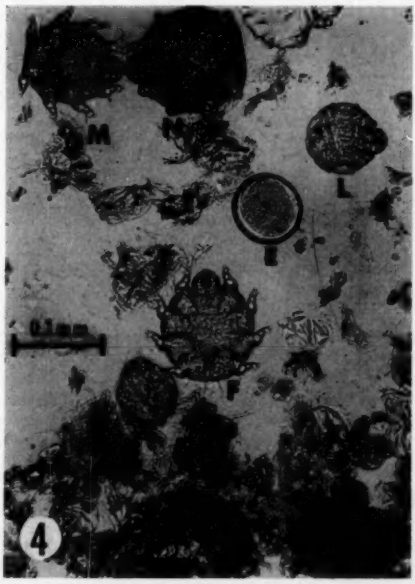


Fig. 4—A pouch dissected to show contents: egg (E); adult female (F); larva (L); adult male (M); nymph (N).

XUM

systemic acaricidal activity. The substitution of laboratory mice infested with *P. simplex* for larger animals infested with other follicle-inhabiting mites reduces the initial cost of the test animals, the cost of maintenance, the amount of space needed, the time required to complete the tests, and the time needed to evaluate the results. Furthermore, the results of controlled studies involving large numbers of mice may be more valid than results obtained from clinical trials on a few larger animals.

SUMMARY

Three weekly topical applications of a 2 per cent aqueous suspension of a wettable powder containing 15 per cent aramite [2-(p-tert-butylphenoxy) isopropyl-2-chloroethyl sulfite] combined with a wetting agent were found to be completely effective against follicular acariasis of mice caused by *Psorergates simplex*.

The use of mice infested with *P. simplex* as test animals for the screening of compounds for topical as well as systemic acaricidal activity was suggested.

References

[1] Flynn, R. J.: Mouse Mange. Proc. Animal Care Panel, *5*, (1954): 96–105.

[2] Flynn, R. J., and Jaroslow, B. N.: Nidification of a Mite (*Psorergates Simplex* Tyrrell, 1883: Myobiidae) in the Skin of Mice. J. Parasitol., *42*, (1956): 49–52.

Further Studies on the Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine

JAMES R. DOUGLAS, Ph.D.; NORMAN F. BAKER, D.V.M., Ph.D.; WILLIAM M. LONGHURST, Ph.D.

Davis and Hopland, California

IN AN EARLIER PAPER,[2] the authors reported experiments which demonstrated that the anthelmintic efficiency of phenothiazine was inversely proportional to the diameter of the particles. In that report, the commercial phenothiazine powder (N. F. grade) referred to as "medium" was estimated to have a mean particle diameter of between 40.0 and 50.0 μ. Further examination of this material, using the air permeation technique, established that the mean particle diameter was actually 12.8 μ.

Whitten[7] reported that, although there were unexplained variations in the anthelmintic efficiency of various samples of phenothiazine against *Trichostrongylus colubriformis*, there was no doubt that the finer samples were superior to the coarser ones.

Gordon[3] confirmed that there appeared to be an inverse relationship between the particle size of phenothiazine and its efficiency against nematode parasites of sheep. He suggested, however, that *Oesophagostomum columbianum* may be less affected by changes in particle size than the trichostrongyles, Ostertagia, Trichostrongylus, and Haemonchus.

Thomas and Elliott[5] subsequently reported that, although finer particles were more efficient against the trichostrongyles of the abomasum and small intestine, larger particles were more efficient against the species in the large intestine (*Oesophagostomum venulosum* and *Chabertia ovina*). They suggested that these results would not be unexpected if anthelmintic activity depended on the presence of phenothiazine in the intestine in the region where the worms were located, since it is reasonable to assume that fine particles would be more rapidly absorbed.

The results of Collier *et al.*[1] demonstrated that the rate of absorption of phenothiazine was at least partially dependent on particle size.

The purpose of these trials was to determine the anthelmintic efficiency of samples of phenothiazine of different mean particle diameters and to construct a curve showing the relationship between particle size and anthelmintic efficiency.

MATERIALS AND METHODS

Although the experiments reported here were conducted at two different times in 1955 and 1956, the methods were the same. Range lambs with natural worm infections were placed in a dry corral and fed alfalfa hay for approximately one month. On the basis of several fecal worm egg counts, using a modified McMaster technique,[4,6] the lambs were allotted to the desired groups, each with essentially the same average egg count. Each group then was treated as desired.

With the exception of a wet-milled product, the phenothiazine was administered in hard gelatin capsules to assure accurate dosage. The wet-milled product was administered with a dose syringe.

On the seventh day after treatment, the lambs were slaughtered and total worm collections were made from the abomasa and small intestines, using methods described previously.[2] The worms were counted and random samples were identified.

In the 1955 experiment, four groups of 10 lambs

TABLE 1—Average Number of Worms per Lamb at Necropsy

	Groups given phenothiazine, 1955				Groups given phenothiazine, 1956				
	Wet-milled				Purified				
Item	1.7 μ*	2.6 μ	16.7 μ	Control	1.9 μ	2.9 μ	10.8 μ	4.8 μ	Control
Abomasum	78	48	528	926	32	59	141	63	634
Small intestine	107	145	421	972	26	210	552	20	1,198
Totals	185	193	949	1,898	58	269	693	83	1,832

*Particle size of phenothiazine.

From the University of California, School of Veterinary Medicine, Davis, and University Field Station, Hopland.

These studies were conducted as a part of Western Regional Research Project W-35, "Internal Parasites of Ruminants."

This work was supported in part by grants from the Dow Chemical Co., Midland, Mich., and the E. I. Du Pont de Nemours and Co., Wilmington, Del. These companies also supplied the phenothiazine used in the investigation.

Acknowledgment is made of the technical assistance of Fay Vance Smalley.

TABLE 2—Percentage Reduction in Number of Worms in Lambs Over Control Group

Item	Groups given phenothiazine, 1955			Groups given phenothiazine, 1956			
	Wet-milled 1.7 μ*	2.6 μ	16.7 μ	1.9 μ	2.9 μ	10.8 μ	Purified 4.8 μ
Abomasum	92	95	43	95	91	78	90
Small intestine	89	85	55	98	83	54	98
Averages	90	90	50	97	85	62	95

*Particle size of phenothiazine.

were treated as follows: Lambs in group 1 were given 25 Gm. of wet-milled phenothiazine, mean particle diameter, 1.7 μ; those in group 2 were given 25 Gm. of phenothiazine N.F. powder, mean particle diameter, 2.6 μ; those in group 3 were given 25 Gm. of phenothiazine N.F. powder, mean particle diameter, 16.7 μ; and those in group 4 were not treated.

In the 1956 experiment, five groups of 7 to 9 lambs each were treated as follows: Lambs in group 1 were given 25 Gm. of phenothiazine N.F. powder, mean particle diameter, 1.9 μ; those in group 2 were given 25 Gm. of phenothiazine N.F. powder, mean particle diameter, 2.9 μ; those in group 3, 25 Gm. of phenothiazine N.F. powder, mean particle diameter, 10.8 μ; those in group 4, 25 Gm. of phenothiazine purified powder, mean particle diameter, 4.8 μ; and those in group 5 were not treated.

The mean particle diameter of the powder samples was determined by the air permeability technique, using a Fisher sub-sieve sizer. The wet-milled product was measured optically.

RESULTS

Numbers of Worms in Lambs at Necropsy.—The average number of worms per lamb recovered at necropsy is shown (table 1). The percentage reduction of worm numbers with reference to the controls is summarized (table 2). Analysis of the data by the "t" test, comparing the wet-milled product (1.7 μ) with the fine powder (2.6 μ), revealed that the differences observed in the abomasa, although small, were significant ($P = 0.005$). The explanation for this apparent contradiction may be found in the different methods of administration of the drug; the wet-milled material could not be given accurately with a dose syringe, and it is probable that the actual dose was somewhat less than 25 Gm. The wet-milled product may have gone directly into the omasum rather than into the rumen, as would be expected in the case of the powder, which might be partially responsible for the difference. It is also possible that the optical measurement of the wet-milled sample did not yield a figure comparable to that obtained on the powdered materials by the air permeation method.

In the 1956 experiment, the difference between the number of worms recovered from the abomasa of lambs in the group given the 2.9 μ N.F. powder and the abomasa of those in the group given the 4.8 μ purified powder was insignificant. This also was true of the worms recovered from the small intestines of lambs in the group given the 1.9 μ N.F. powder and the small intestines of lambs in the group given the 4.8 μ purified powder. All other differences observed were significant, with P values of 0.05 to less than 0.001.

The percentage distribution by species of worms recovered from the abomasa and small intestines of the lambs in the various groups is shown (tables 3, 4). The appearance of *Haemonchus contortus* in 1955, and their absence in 1956, apparently reflects a decrease in spring rainfall on the range where the lambs were raised. These figures do not include either immature Nematodirus sp., which comprised less than 1 per cent of the population, or *Strongyloides papillosus,* which made up

TABLE 3—Percentage Distribution By Species of Nematodes Recovered from the Abomasa of Lambs

Species	Groups given phenothiazine, 1955				Groups given phenothiazine, 1956				
	Wet-milled 1.7 μ*	2.6 μ	16.7 μ	Control	1.9 μ	2.9 μ	10.8 μ	Purified 4.8 μ	Control
O. circumcincta	76	78	83	62	89	79	65	84	84
O. trifurcata	11	11	8	3	11	16	4	11	2
T. vitrinus	12	0	5	4	0	0	27	5	10
T. axei	1	11	4	22	0	5	4	0	4
H. contortus	0	0	0	9	....	....	....	....	....

*Particle size of phenothiazine.

XUM

TABLE 4—Percentage Distribution By Species of Nematodes Recovered from the Small Intestines of Lambs

Species	Groups given phenothiazine, 1955				Groups given phenothiazine, 1956				
	Wet-milled 1.7 μ*	2.6 μ	16.7 μ	Control	1.9 μ	2.9 μ	10.8 μ	Purified 4.8 μ	Control
T. vitrinus	10	8	32	40	70	63	63	0	41
T. colubriformis	3	0	5	1	0	3	2	0	0
N. spathiger	39	21	48	42	0	15	29	100	9
N. filicollis	48	71	15	17	30	19	6	0	50

*Particle size of phenothiazine.

slightly less than 7 per cent of the population in the small intestine in 1956.

DISCUSSION

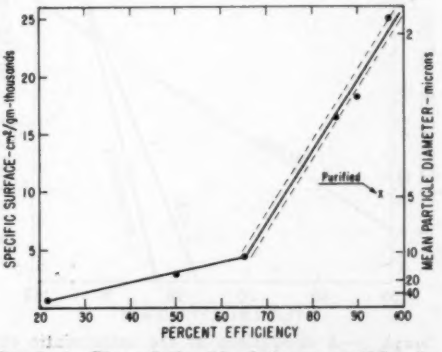
The combined data from the experiment reported previously,[2] plus those from the present trial have been utilized in the construction of the curve shown (graph 1). Since a decrease in particle size is accompanied by a great increase in surface area and if, as the authors have suggested previously,[2] the anthelmintic activity of phenothiazine is dependent on surface contact between the particle and the worm cuticle, then it must follow that there is a direct relationship between surface area and anthelmintic efficiency. When the surface area, expressed as specific surface (sq. cm./Gm.), is plotted on the ordinate as a function of anthelmintic efficiency on the abscissa, it is seen that between the values of aproximately 5,000 sq. cm. per gram and 25,000 sq. cm. per gram there is a linear relationship between these two functions. This portion of the curve (graph 1) has been plotted as a regression line by the method of least squares. The slope of the line through this range represents 619 sq. cm. per gram for each 1 per cent increase in efficiency. The broken lines indicate one standard error of the estimating equation (± 866 sq. cm./Gm.).

When the data within the linear portion of the curve (graph 1) are plotted separately as regression lines representing anthelmintic efficiency in the abomasum and small intestine (graph 2), a highly significant difference is observed ($P = <0.005$). Larger particles are seen to be more effective against the worms in the abomasum than against those inhabiting the small intestine. As the surface area of the phenothiazine increases, this differential effect decreases until efficiency becomes approximately equal in both organs when the mean particle diameter is 2 μ or less (approx. 25,000 sq. cm./Gm.).

The relationship between particle size and anthelmintic efficiency against the species of nematodes encountered in the abomasum and small intestine is illustrated (regression lines in graphs 3, 4). The action of phenothiazine on *Trichostrongylus vitrinus* is not significantly different in either organ ($P = > 0.90$), while *Trichostrongylus axei* and *Ostertagia circumcincta,* which also are not significantly different from each other ($P = 0.02$), appear to be more susceptible than *Nematodirus spathiger* or *Nematodirus filicollis.*

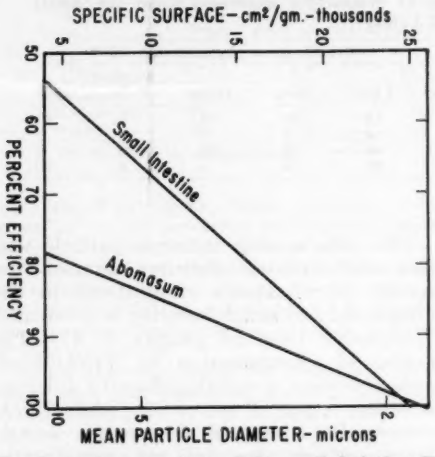
The differences observed (graph 4) apparently are due to chance ($P = 0.10$–0.70); however, it is probable that additional trials would show *T. vitrinus* to be significantly different from *N. spathiger* and *N. filicollis.*

It was reported previously[2] that phenothiazine was, in general, most effective against Ostertagia species, somewhat less effective against Trichostrongylus species, and least effective against Nematodirus species. However, this report failed to take into consideration differences in susceptibility between species; for example, differences between *T. vitrinus* and *T. axei.*



Graph 1—The relationship between particle size and the anthelmintic efficiency of phenothiazine against mixed nematode infections in the abomasa and small intestines of range lambs.

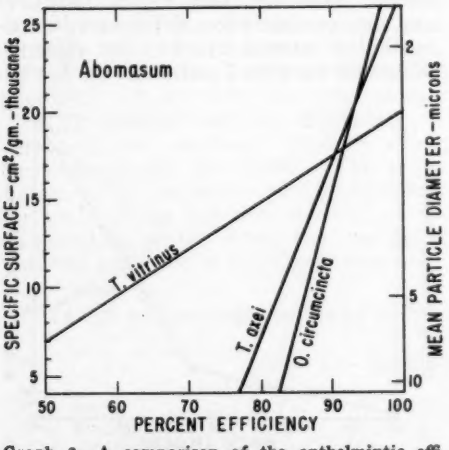
XUM



Graph 2—A comparison of the anthelmintic efficiency of phenothiazine, as a function of particle size, in the abomasa and small intestines of lambs.

This was pointed out by Thomas and Elliott,[5] who found that *T. colubriformis* was more susceptible to phenothiazine than *O. circumcincta*. Unfortunately, in our trials *T. colubriformis* has not occurred in sufficient numbers to permit a valid comparison.

Although the data presented (graph 2) suggest that phenothiazine, particularly with a mean particle diameter of approximately 10 μ, is more efficient in the abomasum than in the small intestine, the results with *T. vitrinus* indicate that the difference is due primarily to reduced efficiency against *N. spathiger* and *N. filicollis*.



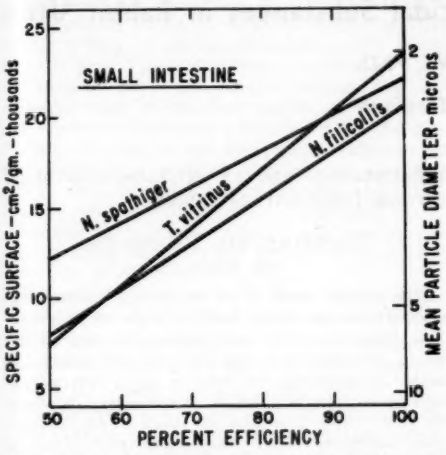
Graph 3—A comparison of the anthelmintic efficiency of phenothiazine, as a function of particle size, against the nematode species in the abomasa of lambs.

The foregoing lends support to the hypothesis set forth previously[2] that the anthelmintic activity of phenothiazine depends on direct solution from the solid state by the cuticle of the worm. However, it does not completely substantiate it, in that an increase in surface area also would increase the potential rate of solution, even though not the final concentration of phenothiazine in a saturated medium. Therefore, it is possible that, if absorption by the host and parasite exceeds the rate of solution so that saturation of the gastroenteric contents is not reached, then the effect of particle size on anthelmintic efficiency could be mediated through a fluid medium as well as cuticular contact with the solid particle.

On the basis of the evidence presented by Gordon[3] and Thomas and Elliott,[5] with respect to the increased efficiency of larger particles of phenothiazine against the nematodes inhabiting the large intestine, it may be reasoned that cuticular contact with a solid particle is the mode of action. Small particles would tend to disappear by solution before they reached the large intestine, leaving only the larger particles.

From the data presented, it appears that a desirable phenothiazine preparation for use in sheep, in areas where Ostertagia, Trichostrongylus, and Nematodirus are the nematodes of greatest importance, should have an average particle diameter of 2 μ or less (specific surface of 25,000 sq. cm./Gm., or higher). The expected anthelmintic efficiency of this preparation would be 90 to 100 per cent.

Unfortunately, the preparation of such a formulation is not without its problems. For example, the basic cost of the phenothiazine would be increased. This, however, probably would be compensated for by the increased efficiency. Possibly the greatest disadvantage would be the increase in bulk, which might not be of great consequence when a drench was used, but which would require an increase in the number of capsules or boluses administered.



Graph 4—A comparison of the anthelmintic efficiency of phenothiazine, as a function of particle size, against the nematode species in the small intestines of lambs.

The apparent greater efficiency of purified phenothiazine as compared with N.F. powder (graph 1) can not be explained on the basis of present information. If further evidence confirms this finding, it may be possible, by using purified phenothiazine with an average particle diameter of approximately 5 μ, to solve the problem of increased bulk without a significant loss of anthelmintic efficiency.

It should be emphasized that these data apply only to sheep; until additional evidence is available it can not be assumed that they apply equally well to cattle.

SUMMARY

There was found to be a linear relationship between specific surface area of phenothiazine preparations and anthelmintic efficiency, within the approximate range of 5,000 to 25,000 sq. cm. per gram. This represents a range of approximately 1 to 10 μ mean particle diameter and 98 to 63 per cent efficiency. Within these limits, an increase in specific surface area of 619 sq. cm. per gram resulted in a 1 per cent increase in efficiency.

The anthelmintic efficiency of a purified phenothiazine preparation with a mean particle diameter of 4.8 μ was found to be approximately equal to that of N.F. material with a mean particle diameter of 1.9 μ.

References

[1] Collier, H. B., Allen, D. E., and Swales, W. E.: Observations of the Fate of Phenothiazine in Domestic Animals. Canad. J. Res., *21*, (1943): 151–159.

[2] Douglas, J. R., Baker, N. F., and Longhurst, W. M.: The Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine. Am. J. Vet. Res., *17*, (April, 1956): 318–323.

[3] Gordon, H. McL.: The Influence of Particle Size on the Anthelmintic Efficiency of Phenothiazine in Sheep. Austral. Vet. J., *32*, (1956): 258–268.

[4] Gordon, H. McL., and Whitlock, H. V.: A New Technique for Counting Nematode Eggs in Sheep Faeces. J. Council Scient. Indust. Res. (Australia), *12*, (1939): 50–52.

[5] Thomas, P. L., and Elliott, D. C.: The Use of Fine-Particle Phenothiazine Against *Trichostrongylus Colubriformis* in Sheep with Observations on Its Use Against Other Species of Worms. New Zealand Vet. J., *5*, (1957): 66–69.

[6] Whitlock, H. V.: Some Modifications of the McMaster Helminth Egg-Counting Technique and Apparatus. J. Council Scient. Indust. Res. (Australia), *21*, (1948): 177–180.

[7] Whitten, L. K.: The Efficiency of Phenothiazine Against *Trichostrongylus Colubriformis* in Sheep. New Zealand Vet. J., *4*, (1956): 63–68.

XUM

Investigations Concerning Bactericidal Substances in Rabbit Uteri

H. W. HAWK, Ph.D.

Beltsville, Maryland

UTERI OF ANIMALS in the estrous phase of the reproductive cycle possess a highly efficient defense mechanism.[1, 4-6, 14] The migration of leukocytes into the uterine lumen after experimental inoculation is associated closely with bactericidal activity of the uterus.[4] Phagocytosis, however, may not be the principal uterine defense mechanism in these circumstances; inflammatory exudates from estrous rabbit uteri were found to have bactericidal properties after the leukocytes had been removed.[4] The bactericidal substances in these exudates were active against *Escherichia coli in vitro*, as well as in inoculated uteri of pseudopregnant rabbits.

The degree of bactericidal activity of leukocyte-free inflammatory exudates was dependent largely on the hormonal state of the animal; exudates from pseudopregnant rabbit uteri were much less bactericidal *in vitro* than those from estrous rabbit uteri,[4] reflecting the difference in bactericidal activity of their uteri. The uterine defense mechanism of luteal-phase rabbits and cows is much less efficient than that of estrous-phase animals,[1, 2, 4-6, 14] this difference possibly being related to the observation that the inflammatory response to experimental inoculation develops more slowly in pseudopregnant than in estrous rabbit uteri.[4]

This study was undertaken to investigate some possible sources of the bactericidal factors in inflammatory uterine exudates. Blood serum is known to possess bactericidal properties and, thus, might be suspected as a source of the bactericidal factors in the uterine lumen. Since serum bactericidins which kill gram-negative bacteria are labile when heated at 56 C. for 30 minutes,[16] a comparison was made of the thermostability at this time and temperature of the bactericidal substances in inflammatory uterine exudates and blood serums from estrous rabbits.

From the Dairy Cattle Research Branch, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

The author thanks Dr. J. F. Sykes, for counsel during the course of the study and preparation of the manuscript; and G. D. Turner and R. D. Gaalaas, for technical assistance.

EXPERIMENTAL PROCEDURES AND RESULTS

The rabbits used in all these experiments were sexually mature Dutch females, eight to 12 months old. Estrous rabbits were in heat the day of use, being receptive to bucks, but were not allowed to mate. Pseudopregnant rabbits were obtained by mating estrous females to vasectomized bucks or by intravenous injection of 100 I.U. of chorionic gonadotrophin. They were used eight, nine, or ten days after the induction of ovulation.

Experiment 1—Relative Thermostability of Bactericidal Factors in Blood Serum and Uterine Inflammatory Exudate.—Eight estrous rabbits were used in this experiment; pseudopregnant rabbits were not included because their uterine exudates had relatively limited bactericidal activity in comparison with those from estrous rabbits four hours after uterine inoculation.[4] Blood was drawn aseptically from the heart of each rabbit to obtain serum.

The rabbit was laparotomized under pentobarbital sodium (Nembutal*) anesthesia and each uterine horn was ligated at the tubouterine junction and cervix, a ligature being placed around the posterior end of each cervix through an incision in the anterior vagina. Each uterine horn was inoculated by puncturing the uterine wall carefully with a 26-gauge hypodermic needle and introducing 0.2 ml. of a saline solution (0.87% NaCl) suspension of a 24-hour culture of *Esch. coli* washed from trypticase soy agar slants. Particular care was taken not to puncture veins during inoculation, as this could have resulted in an undesired seepage of blood into the uterine lumen.

Four hours after inoculation, the rabbit was killed and each uterine horn was flushed as thoroughly as possible with 1.0 ml. of sterile physiological saline solution. The flushings from the two horns were pooled and the leukocytes removed by centrifugation at 600 r.p.m. for five minutes (19.5-cm. radius). The supernantant fluid (leukocyte-free exudate) was divided equally and one sample was heated at 56 C. for 30 minutes in a constant-temperature water bath, as was 0.5 ml. of blood serum. The heated and unheated samples of exudate and serum, as well as 0.5 ml. of saline solution, were inoculated with 0.2 ml. of a freshly prepared saline solution suspension of *Esch. coli* and incubated at 37 C. for two hours.

* Nembutal is produced by Abbott Laboratories, North Chicago, Ill.

XUM

The incubation mediums were diluted serially and duplicate 1.0-ml. aliquots of several tenfold dilutions were mixed in Petri dishes with violet red bile agar. The plates were incubated at 37 C. for 18 to 24 hours, the colonies counted, and calculations made of the total number of live *Esch. coli* recovered from each incubation tube. Several 0.01-ml. aliquots of the first tenfold dilution of each incubation medium were spread carefully over a circular area 1 cm. in diameter and dried, and the residues were stained and examined microscopically to determine whether any bactericidal activity of the exudates and serums was associated with lysis of the bacterial cells.

The pH of each rabbit's exudate was determined by use of pH indicator paper or a pH meter.

The mean log number of *Esch. coli* alive in each medium after the incubation period (table 1) was: heated blood serum, 8.57 (372,000,000); unheated blood serum, 5.36 (229,000); heated uterine exudate, 5.28 (191,000); unheated uterine exudate, 4.62 (41,700); and in saline solution, 8.17 (148,000,000). The uterine exudates were highly bactericidal *in vitro* and retained strong bactericidal properties after being heated at 56 C. for 30 minutes. Unheated blood serum also was highly bactericidal *in vitro*, but the factors in serum which killed *Esch. coli* were inactivated by the heat treatment; bacterial growth occurred in all the heated serum samples.

Statistically, the numbers of *Esch. coli* alive after incubation in the heated exudates, unheated exudates, and unheated serums were each significantly less ($P = <0.01$) than the number alive in saline solution, and the log difference between the numbers of *Esch. coli* alive in heated and unheated blood serum was significantly greater ($P = <0.01$) than the difference between the numbers alive in heated and unheated uterine exudates.

The decisive differential effect of heating suggested that the bactericidal substances in inflammatory uterine exudates of estrous rabbits were not identical to those in blood serum. The bactericidal action of the substances in exudates and serums also appeared to differ. Microscopic examination of the dried residue of the incubation mediums showed that the bacterial cells incubated in unheated blood serum had been lysed, while most of those incubated in either heated or unheated uterine exudates remained intact, although killed.

The bactericidal substances in the exudates were not thermostable, since heating at 100 C. for ten minutes destroyed their bactericidal properties.

Acidity or alkalinity did not appear to be significant factors with regard to the bactericidal activity of the uterine exudates. The exudates varied from neutrality only slightly as they were flushed from the uteri and were invariably slightly alkaline (pH 7.1–7.8), being similar in this respect to the slight alkalinity of exudates from the pleural cavities of dogs during the early stages of experimental inflammation.[13]

Uteri of all the rabbits in this experiment showed a severe inflammatory response four hours after inoculation. Except for the rabbit in replicate 5, the total number of leukocytes in the uterine lumen of each rabbit was over 100 million. Since a highly bactericidal leukocyte-free exudate is invariably associated with a pronounced leukyocytic influx, leukocytes were investigated as a possible source of the bactericidal factors in inflammatory uterine exudates.

Experiment 2—Bactericidal Activity of Leukocytic Extracts.—Each uterine horn of 6 estrous rabbits was inoculated with *Esch. coli* and the rabbits were killed four hours later. Each horn

TABLE 1—Log Numbers of Escherichia Coli Alive After Incubation in Vitro for Two Hours in Heated* and Unheated Blood Serum and Inflammatory Uterine Exudate of Eight Estrous Rabbits

Rabbit (No.)	Inoculum per incubation tube	*In vitro* incubation medium				
		Blood serum		Uterine exudate †		Saline solution
		Heated	Unheated	Heated	Unheated	
1	8.11	8.49	5.29	4.00	3.60	8.01
2	8.09	8.40	5.56	4.59	3.48	8.02
3	8.42	8.59	5.58	6.25	5.44	8.33
4	8.32	8.59	5.73	6.32	5.35	8.28
5	8.35	8.57	5.28	6.86	6.33	8.27
6	8.21	8.56	4.78	3.70	3.40	8.10
7	8.19	8.79	5.29	5.59	5.11	8.12
8	8.39	8.56	5.37	4.88	4.24	8.23
Mean	8.26	8.57	5.36	5.28	4.62	8.17

* At 56 C. for 30 minutes. † Each uterine horn was inoculated with *Esch. coli* four hours before the uterine exudate was collected; the mean log number of *Esch. coli* injected per horn was 8.31 (204,000,000).

was flushed aseptically with 1.0 ml. of sterile physiological saline solution, the flushings from the horns of each rabbit were pooled, and leukocytes were collected by centrifugation. The supernatant fluids (leukocyte-free exudates) were saved and tested for bactericidal activity. The volume of cellular debris, consisting mostly of polymorphonuclear leukocytes, varied between 0.05 and 0.35 ml. for each rabbit.

The leukocytes from each rabbit were washed four times in 10 ml. of sterile saline solution, being removed from the suspending medium each time by centrifugation. The washed leukocytes were disrupted by grinding with sand in a mortar containing approximately 2 ml. of saline solution. Sand and cellular debris were removed by centrifugation, the supernatant fluid (leukocytic extract) was divided equally, and one sample was heated at 56 C. for 30 minutes to test for thermostability at this time and temperature of any bactericidal substances in the extracts. The heated and unheated leukocytic extracts, the original supernatant fluid from the uterine flushings, and 1 ml. of saline solution were inoculated with *Esch. coli* and incubated at 37 C. for two hours.

A loopful of luminal contents was taken from the uterus of each rabbit before flushing and was dried, stained, and examined microscopically to learn the condition of the leukocytes in the uterus. The pH of the leukocytic extracts was determined by the use of pH indicator or a pH meter. Uterine exudates and leukocytic extracts from other estrous rabbits were tested for bactericidal activity after heating at various temperatures above 56 C. by inoculating the heated sample, an unheated sample, and saline solution with approximately 200 million *Esch. coli* cells each and incubating for two hours.

The pH of the leukocytic extracts was 4.5 to 5.0. Extracts were therefore obtained from leukocytes recovered from inoculated uteri of an additional 6 estrous rabbits, the extract of each rabbit's leukocytes was divided equally, and one sample was neutralized with 0.15 N NaOH to determine whether the extracts possessed bactericidal properties independent of their acidity. Each sample was inoculated with approximately 200 million *Esch. coli* and incubated at 37 C. for two hours.

The mean log numbers of *Esch. coli* alive in the indicated mediums after the incubation period (table 2) are: leukocytic extracts, 5.36 (229,000); heated leukocytic extracts, 5.89 (776,000); leukocyte-free exudates, 4.97 (93,300); and saline solution, 8.12 (132,000,000). The mean numbers of *Esch. coli* alive after incubation in the heated and unheated leukocytic extracts and in the leukocyte-free exudates were each significantly less ($P = <0.01$) than the number alive in saline solution. The results indicate that leukocytes recovered from inoculated uteri of estrous rabbits, as well as the leukocyte-free exudates from these rabbits, contain one or more substances which are highly bactericidal to *Esch. coli*. The bactericidal substances in the leukocytic extracts were affected only slightly by heating at 56 C. for 30 minutes, and the bactericidal action of the extracts was not associated with appreciable lysis of bacterial cells.

Neutralization of leukocytic extracts did not eliminate their bactericidal activity, indicating that the extracts possessed bactericidal properties independent of their acidity. Mean log numbers of *Esch. coli* alive after incubation in each of six unneutralized extracts were: 6.16 (1,450,000); in each of six neutralized extracts, 7.05 (11,200,000); and in saline solution, 8.13 (135,000,000). The differences in numbers of *Esch. coli* alive after incubation in the neutralized extracts and in saline solution were significant statistically ($P = 0.03$).

As far as was determined during this experiment, the bactericidal substances in leukocytic extracts and leukocyte-free exudates possessed similar properties of thermostability. The bactericidal activity of both the exudates and extracts was diminished slightly after heating at 56 C. for 30 minutes (tables 1, 2), and progressively more bactericidal activity was lost by the heating of highly bactericidal exudates and extracts at 60, 70, and 80 C. for 30 minutes, although considerable activity

TABLE 2—Log Numbers of Escherichia Coli Alive After Incubation in Vitro for Two Hours in Extracts of Leukocytes and Leukocyte-Free Exudates from Six Estrous Rabbit Uteri

Rabbit (No.)	Inoculum per incubation tube	*In vitro* incubation medium			
		Leukocytic extracts	Heated * leukocytic extracts	Uterine exudates	Saline solution
1	8.31	5.03	5.59	3.34	8.14
2	8.17	4.48	4.83	5.13	8.03
3	8.18	3.19	4.94	3.57	8.14
4	8.28	6.02	5.89	4.88	8.12
5	8.28	7.41	7.51	6.88	8.10
6	8.31	6.01	6.57	6.01	8.18
Mean	8.26	5.36	5.89	4.97	8.12

* At 56 C. for 30 minutes.

still was present after heating at 80 C. Only slight, if any, activity was observed after heating at 90 C. for 30 minutes or 100 C. for ten minutes.

Although *Esch. coli* were incubated routinely for two hours in exudates and extracts, highly bactericidal samples of exudates and extracts killed most of 200 million *Esch. coli* in 15 minutes and killed over 99.9 per cent in one hour.

Again, a definite association was observed between the volume of cells in the uterine flushings and the bactericidal activity of the leukocyte-free exudates. A similar association was observed, as would be expected, between the volume of cells in the flushings and the bactericidal activity of the leukocytic extracts.

The results of this experiment suggest that the bactericidal factors in leukocyte-free uterine exudate could come from leukocytes. Within the limits of the methods used, no differences were observed in the properties of the bactericidal factors in the uterine exudates and leukocytic extracts. Leukocytic extracts were highly bactericidal to *Esch. coli*, as were the exudates, neither was decidedly bacteriolytic, and the substances in each possessed similar properties of thermostability. Further, microscopic examination of cellular exudates taken directly from the uteri showed that a small percentage of the polymorphonuclear leukocytes was obviously damaged, and occasional cellular fragments were seen. It seems likely that the bactericidal substances extractable from leukocytes would diffuse into the uterine luminal fluid from these damaged cells and might account, wholly or partly, for the bactericidal properties of the leukocyte-free exudate. Release of such substances by intact leukocytes might also be considered.

One other possibility was considered with regard to possible sources of the bactericidal factors in uterine inflammatory exudates, *i.e.*, that such factors were associated directly with the bacterial inoculum used to induce the inflammation and were not produced by the rabbit. A nonbacterial irritant therefore was used to induce uterine inflammation and the resulting exudates were tested for bactericidal activity.

Experiment 3—Bactericidal Activity of Silica-Induced Inflammatory Exudates.—Uteri of 10 estrous and 5 pseudopregnant rabbits were injected with silica. Each uterine horn from the first 5 estrous rabbits was ligated and injected with 0.2 ml. of a sterile saline solution suspension of washed, finely ground sand; these rabbits were killed four hours later. One estrous and 1 pseudopregnant rabbit were paired in each of the next five replicates. Each uterine horn from these rabbits was injected with 0.2 ml. of a sterile saline solution suspension of washed diatomaceous silica. Eight rabbits (replicates 6 through 9; table 3) were killed six hours after injection; the rabbits in replicate 10 were killed 16 hours after injection. Each uterine horn was flushed with 1.0 ml. of sterile physiological saline solution, the flushings of the two horns were pooled, and the leukocytes and silica were removed by centrifugation. The sediment was examined for cellular content. The supernatant fluids (leukocyte-free exudates) and equal volumes of saline solution were inoculated with *Esch. coli* and incubated for two hours at 37 C., and the number of *Esch. coli* alive after the incubation period was determined. To test for any bactericidal factors associated with the silica itself, an aliquot of saline solution in which silica had been kept for at least 24 hours was inoculated and incubated along with each replicate of exudates.

TABLE 3—Log Numbers of Escherichia Coli Alive After Incubation in Vitro for Two Hours in Leukocyte-Free Silica-Induced Inflammatory Exudates of Ten Estrous and Five Pseudopregnant Rabbit Uteri

Replicate	Inoculum per incubation tube	Estrous rabbit exudates *	Pseudopregnant rabbit exudates *	Saline solution
1	8.12	4.18	.....	8.01
2	8.31	5.77	.....	8.24
3	8.29	7.81	.....	8.17
4	8.24	6.15	.....	8.11
5	8.44	8.57	.....	8.22
6	8.38	6.50	8.45	8.30
7	8.37	7.47	8.55	8.30
8	8.36	6.17	6.72	8.27
9	8.35	6.70	8.55	8.24
10	8.28	5.68	6.51	8.21
Mean	8.31	6.50	7.76	8.21

*All exudates except those in the first three replicates were heated at 56 C. for 30 minutes before inoculation. Exudate-donor rabbits in the first five replicates were killed four hours, those in the next four replicates, six hours, and those in the last replicate, 16 hours after silica injection.

All exudates except those from the first 3 estrous rabbits (replicates 1, 2, 3) were heated for 30 minutes at 56 C. before inoculation.

The mean log numbers of *Esch. coli* alive after the incubation period (table 3) were: in estrous rabbit exudate, 6.50 (3,160,000); pseudopregnant rabbit exudate, 7.76 (57,500,000); and saline solution, 8.21 (162,000,000). The number of *Esch. coli* alive after incubation in the estrous rabbit exudates was significantly less than the number alive after incubation in saline solution ($P = <0.01$); exudates from 9 of the 10 estrous rabbits were bactericidal, as were exudates from 2 of the 5 pseudopregnant rabbits. Washed silica itself apparently possessed no bactericidal properties, since the numbers of *Esch. coli* alive after incubation in saline solution from which silica had been removed did not differ from the numbers alive in the other saline solution controls; therefore, only one set of saline solution data is listed (table 3).

The results of this experiment indicate that a bactericidal inflammatory exudate can be produced by the uterus in response to a nonbacterial irritant, thus failing to suggest that factors associated with the inoculum itself were primarily responsible for the bactericidal activity of *Esch. coli*-induced exudates.

It can not be stated with certainty that the bactericidal substances in silica-induced inflammatory exudates were identical to those in *Esch. coli*-induced exudates, although they were similar as far as they were tested. The bactericidal substances in the silica-induced exudates were stable after heating at 56 C. for 30 minutes, were inactivated by heating at 100 C. for ten minutes, and apparently were not greatly bacteriolytic *in vitro*.

In general, bactericidal activity of the uterine exudates was related to the degree of inflammatory response to the silica. Exudates that failed to show bactericidal activity were from uteri that failed to show leukocytic responses. The pseudopregnant rabbits did not respond as quickly or as consistently to the irritant as the estrous rabbits, this difference being similar to the differential leukocytic response to *Esch. coli* during the first four hours postinoculation.[4]

Exudates from the estrous rabbits in the last five replicates were more bactericidal than those from the pseudopregnant rabbits ($P = <0.02$). This difference is similar to that between *Esch. coli*-induced exudates from rabbits of the two endocrine states.[4]

DISCUSSION

These experiments have demonstrated that extracts of the cellular component of the uterine inflammatory response contained bactericidal substances which were similar to those in leukocyte-free inflammatory uterine exudates, indicating that leukocytes may be the source of the bactericidal factors in the exudates. The close association between the degree of leukocytic response and the bactericidal activity of uterine exudates also is suggestive. The presence of bactericidal substances in inflammatory uterine exudates thus may be a consequence of leukocytic influx. Diffusion from damaged leukocytes might account for the presence of these substances in the exudates, and perhaps the possibility should not be overlooked that intact leukocytes might be capable of releasing such factors under certain conditions. Recent experimental results indicated that leukocytes were able to release pyrogen into the surrounding medium while intact and viable.[11] Further experimentation will be necessary to establish more firmly or to disprove the suggestion that bactericidal substances in uterine exudates are derived from leukocytes.

These experiments have not ruled out the possibility that the uterine tissue itself produced a part of the bactericidal factors in the inflammatory exudates.

Occasionally, a uterine exudate from an estrous rabbit has failed to show bactericidal activity *in vitro*, although few of the introduced bacteria were recovered alive from the uterine lumen. In all such instances, relatively few leukocytes (<10 million) were flushed from the uterus. Only slight bactericidal potential of such exudates might be difficult to detect in the presence of growth-promoting nutritive substances.

Several investigators have reported the bactericidal activity of leukocytic extracts.[16] The one or more bactericidal substances in the leukocytic extracts in this study, and possibly those in the inflammatory uterine exudates as well, may be identical to a bactericidal substance extracted recently from leukocytes induced

XUM

into the peritoneal cavities of rabbits.[8,9] The substance was called "phagocytin." It was particularly bactericidal to gram-negative enteric bacilli, but was not bacteriolytic. It was stable to heating at 56 C. for 30 minutes and its bactericidal activity was destroyed by heating at 100 C. for ten minutes. The bactericidal substances in the uterine exudates and leukocytic extracts in this study resemble phagocytin in these respects.

Although polymorphonuclear leukocytes are known to contain lysozyme, this substance ordinarily is not bactericidal to *Esch. coli*[8,15] and apparently is not identical to the bactericidal factors in the exudates and extracts in this study. Hemoglobin is bactericidal to *Esch. coli* under certain conditions,[10] but few erythrocytes could be found in the cellular exudates in this study, and the color of the leukocyte-free exudates was not indicative of the presence of hemoglobin.

If the bactericidal substances in uterine exudates do originate in leukocytes, the differential rate of leukocytic influx into inoculated uteri of estrous and pseudopregnant rabbits might explain the difference in bactericidal activity of their exudates during the early stages of inflammation. The number of leukocytes in the uterine lumina of pseudopregnant rabbits four hours after inoculation was approximately 5 per cent as great as the number in estrous rabbit uteri, and uterine exudates from the estrous rabbits were much more bactericidal than those from pseudopregnant rabbits.[4] A similar difference between estrous and pseudopregnant rabbits in the rate of leukocytic influx and bactericidal activity of uterine exudates was observed in the silica-inoculated uteri in this study.

At later stages of experimentally induced uterine inflammation, luteal-phase rabbit and cow uteri contain greater amounts of pus than do uteri of estrous-phase animals.[1-3,12,14] If the association between the degree of leukocytic influx and the bactericidal activity of leukocyte-free exudates is independent of the hormonal state of the animal, it might follow that exudates from pseudopregnant rabbits would be more bactericidal at later stages of uterine inflammation than those from estrous rabbits. In an experiment subsequent to those reported here, this was found to be the case; 16 hours after inoculation of ligated uteri with *Esch. coli*, leukocyte-free exudates from pseudopregnant rabbits were extremely bactericidal—even more so than exudates from estrous rabbits at the same stage of inflammation.[7]

These experiments were not intended to elucidate the importance to the uterine defense mechanism of bactericidal substances in inflammatory uterine exudates. However, the apparent lack of lytic action by the bactericidal substances *in vitro* would make questionable the possibility that these substances constitute the only uterine defense mechanism against introduced *Esch. coli*, since, after the action of an effective defense mechanism, few of the introduced bacteria can be found in the uterine flushings of estrous rabbits. Thus, it seems likely that other defenses also are active, although these bactericidal substances may be an integral part of the uterine defense mechanism.

SUMMARY

Investigations were made on possible sources of bactericidal substances in leukocyte-free inflammatory exudates of rabbit uteri.

Bactericidal substances in cell-free exudates from *Escherichia coli*-inoculated estrous rabbit uteri were stable when heated at 56 C. for 30 minutes. Blood serum lost its bactericidal activity under the same treatment, indicating that the bactericidal substances in blood serum and in uterine inflammatory exudates were not identical.

Extracts of washed leukocytes collected from inoculated uteri of estrous rabbits were highly bactericidal to *Esch. coli*. The leukocytic extracts possessed bactericidal properties independent of their acidity. The bactericidal substances in the leukocytic extracts and in leukocyte-free uterine exudates were similar as far as they were tested; they were relatively thermostable, but were destroyed by heating at 100 C. for ten minutes, and were not bacteriolytic.

Leukocyte-free exudates obtained from silica-injected estrous rabbit uteri were also bactericidal. A nonbacterial irritant thus was able to induce the presence of bactericidal substances in the uterus, indicating that factors not associated with the bacterial inoculum itself were responsible for the bactericidal activity of *Esch. coli*-induced exudates. Silica-induced exudates

XUM

from pseudopregnant rabbits were less bactericidal than those from estrous rabbits.

Within the limits of the methods used in these experiments, the results support the hypothesis that leukocytes are the principal source of bactericidal substances in leukocyte-free inflammatory uterine exudates. The presence of such substances in the exudates might be due to diffusion from damaged leukocytes or to release by intact leukocytes.

References

[1] Black, W. G., Simon, J., Kidder, H. E., and Wiltbank, J. N.: Bactericidal Activity of the Uterus in the Rabbit and the Cow. Am. J. Vet. Res., *15*, (April, 1954): 247–251.

[2] Black, W. G., Simon, J., McNutt, S. H., and Casida, L. E.: Investigations on the Physiological Basis for the Differential Response of Estrous and Pseudopregnant Rabbit Uteri to Induced Infection. Am. J. Vet. Res., *14*, (April, 1953): 318–323.

[3] Black, W. G., Ulberg, L. C., Kidder, H. E., Simon, J., McNutt, S. H., and Casida, L. E.: Inflammatory Response of the Bovine Endometrium. Am. J. Vet. Res., *14*, (April, 1953): 179–183.

[4] Hawk, H. W.: The Influx of Leukocytes and Presence of Bactericidal Substances in Inoculated Uteri of Estrous and Pseudopregnant Rabbits. J. Anim. Sci., *17*, (1958): 416.

[5] Hawk, H. W., Simon, J., Cohen, H., McNutt, S. H., and Casida, L. E.: The Relative Bactericidal Activity of the Uterine and Body Cavities of Estrous and Pseudopregnant Rabbits. J.A.V.M.A., *126*, (April, 1955): 268–270.

[6] Hawk, H. W., Simon, J., McNutt, S. H., and Casida, L. E.: Investigations on the Endocrine-Controlled Defense Mechanism of Estrous and Pseudopregnant Rabbit Uteri. Am. J. Vet. Res., *18*, (Jan., 1957): 171–173.

[7] Hawk, H. W., and Sykes, J. F.: Bactericidal Activity of Inflammatory Exudates from Rabbit Uteri. Abstr. in J. Dai. Sci., *41*, (1958): 739.

[8] Hirsch, J. G.: Phagocytin: A Bactericidal Substance from Polymorphonuclear Leukocytes. J. Exptl. Med., *103*, (1956): 589.

[9] Hirsch, J. G.: Studies of the Bactericidal Action of Phagocytin. J. Exptl. Med., *103*, (1956): 613.

[10] Hobson, D., and Hirsch, J. G.: The Antibacterial Activity of Hemoglobin. J. Exptl. Med., *107*, (1958): 167.

[11] King, M. K., and Wood, W. B., Jr.: Studies on the Pathogenesis of Fever. III. The Leukocytic Origin of Endogenous Pyrogen in Acute Inflammatory Exudates. J. Exptl. Med., *107*, (1958): 279.

[12] McDonald, L. E., Black, W. G., McNutt, S. H., and Casida, L. E.: The Response of the Rabbit Uterus to Instillation of Semen at Different Phases of the Estrous Cycle. Am. J. Vet. Res., *13*, (July, 1952): 419–424.

[13] Menkin, V.: Studies on Inflammation. X. The Cytological Picture of an Inflammatory Exudate in Relation to Its Hydrogen Ion Concentration. Am. J. Path., *10*, (1934): 193.

[14] Rowson, L. E. A., Lamming, G. E., and Fry, R. M.: The Relationship Between Ovarian Hormones and Uterine Infection. Vet. Rec., *65*, (1953): 335.

[15] Salton, M. R. J.: The Properties of Lysozyme and Its Action on Microorganisms. Bact. Rev., *21*, (1957): 82.

[16] Skarnes, R. C., and Watson, D. W.: Antimicrobial Factors of Normal Tissues and Fluids. Bact. Rev., *21*, (1957): 273.

Histological Observations of the Genitalia of the Immature, the Castrated, and the Mature Bovine Male

D. M. TROTTER, D.V.M., M.S.

Manhattan, Kansas

ANATOMISTS, histologists, and pathologists have assumed for many years that the microscopic structure of the male bovine genitalia was almost identical to that of man. In the literature there are many good and complete descriptions of the histology of the human male genitalia, but no similar complete descriptions for the bovine male genitalia were found.

REVIEW OF LITERATURE

Many of the recorded studies dealt only with one organ or structure of the system, and under special circumstances, as in the study of the interstitial cells of the testis during prenatal life by Hooker,[23] observations on the erectile tissue by Deysack,[6] and the study of the blood supply of the testis by Harrison.[18]

Numerous studies on sterility included isolated areas of the system under abnormal conditions, either natural or experimental, but did not include the normal histology of the whole system.[4, 7, 13, 16, 20, 21, 24]

Numerous other studies of small areas or of the system under special circumstances included a study of congenital abnormalities by Rollinson;[28] bacteriological, clinical, and pathological studies by Easley *et al.*[9] and Kimball *et al.*;[25] the histology of the male genital system as related to the other endocrine organs of the body by Cupps *et al.*;[6] and histological studies of small pathological areas for diagnosis of clinical conditions by Perkins *et al.*,[22] Fossland,[11] and Milne.[19]

Phillips and Andrews [25] made an age-development study, but concentrated their work on the spermatozoa; Williams [41] described testicular atrophy, but gave little normal histology. Many other studies described the basic normal or abnormal development of the organs or cells with special reference to certain cells or areas.[2, 3, 25, 27, 41, 42]

A comparison of the glandular accessory organs in wild and domestic animals was recorded by Macklin and Macklin.[26] Melampy *et al.*[20] made a complex study of the composition and histochemistry of the testicle. Cellular and secreted products of the testes have been the subject of many studies.[1, 10, 19, 31, 34]

The effect of castration on urethral development was studied by Marsh and Safford,[27] but effects on the other parts of the system were not included.

Haq [17] recorded the most complete study of the epithelial structures of the male bovine genital tract but did not include the whole system in his study. He mentioned the lack of studies on the normal organs as a handicap in the study of abnormal or pathological conditions.

The gross anatomy textbooks of McLeod [29] and Sisson and Grossman [39] gave accurate descriptions of the gross structures of the male bovine genital system but limited any histological information to brief footnotes or statements within the formal descriptions.

The formal textbooks on histology were nearly all based on human structure. A few isolated statements on the male bovine genitalia were present in some works.[5, 14, 15, 25, 36]

The textbooks on veterinary histology were devoted primarily to domestic animals in general and made only brief reference to the bovine male genitalia.[12, 40]

This study was conducted in an effort to confirm or disprove assumed facts, to find new information, and to consolidate the information regarding the similarity of the microscopic structure of the genitalia of man and the bovine male.

MATERIALS AND METHODS

Four 1-day-old calves, 5 animals killed at a slaughterhouse, 7 animals used for gross anatomical study, and 4 mature bulls killed for necropsy were used in this study. The complete genital system, including the testicles, prostate gland, seminal vesicles, bulbourethral glands, penis, prepuce, scrotum, and accessory muscles, was collected from each of the animals.

The specimens were preserved in a 10 per cent solution of formaldehyde. Latex injections in the arterial system were made to facilitate gross dissection and study. Tissue specimens from 27 different areas of each set of organs were collected for microscopic study. The tissue specimens were dehydrated with alcohols and then infiltrated, embedded, and blocked in paraffin. Sections were cut with the microtome at 5 to 7 μ and stained with hematoxylin and eosin. Photomicrographs were made of the areas most important to the histological study.

OBSERVATIONS

THE TESTICLES

The surface of the testicle was covered by the tunica vaginalis propria, except at the attachment of the epididymis. This tunic

From the Department of Anatomy, School of Veterinary Medicine, Kansas State College, Manhattan.

XUM

was basically a single layer of mesothelial cells. The tunica albuginea consisted of a layer of connective tissues just beneath the tunica vaginalis. It was composed mostly of collagenous fibers; some elastic fibers were evident.

Small, fragile connective tissue septa extended into the gland from the tunica albuginea to a more dense and concentrated area of connective tissue that formed a longitudinal core through the center of the organ. This core was designated as the mediastinum by Maximow and Bloom.[28] The septa divided the gland into indistinct lobules. The convoluted seminiferous tubules were found within the lobules.

Between the seminiferous tubules were the interstitial tissue and the interstitial cells (Leydig cells). The interstitial tissue was a continuation and modification of the connective tissue of the small supporting septa. The interstitial tissue had decreased fibrous tissue elements and increased vascular elements, as compared to the structure of the septa. The interstitial cells were located in irregular areas between the seminiferous tubules.

The cells appeared to be modifications of the connective tissue cells of the area. The round-to-oval basophilic nucleus was the prominent feature of the oval- to irregular-shaped interstitial cell. The production of male sex hormone has been suggested as the prime function of the cells.[28]

The epithelium of the seminiferous tubules rested on a basement membrane which, in turn, rested on the interstitial tissue (fig. 1). In the tubules of the mature animal, two basic cell types, the sustentacular cells and the spermatogenic cells, were present.

The sustentacular cells (Sertoli cells) were relatively few in number as compared with the spermatogenic cells. The sustentacular cells rested on the basement membrane and extended toward the lumen of the seminiferous tubule. The nuclei were basically round to oval and stained lighter than the nuclei of the various stages of the spermatogenic cells. The cytoplasm of the sustentacular cells was very irregular and often indistinct, depending on the extent of the characteristic crowding by the spermatogenic cells.

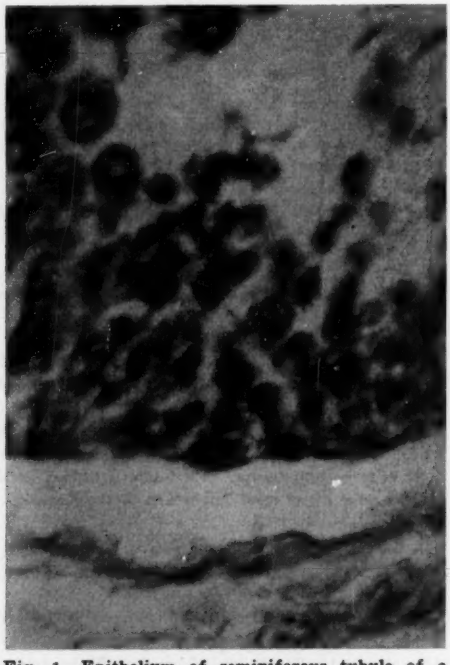


Fig. 1—Epithelium of seminiferous tubule of a mature testicle of the bull. H & E stain; x 380.

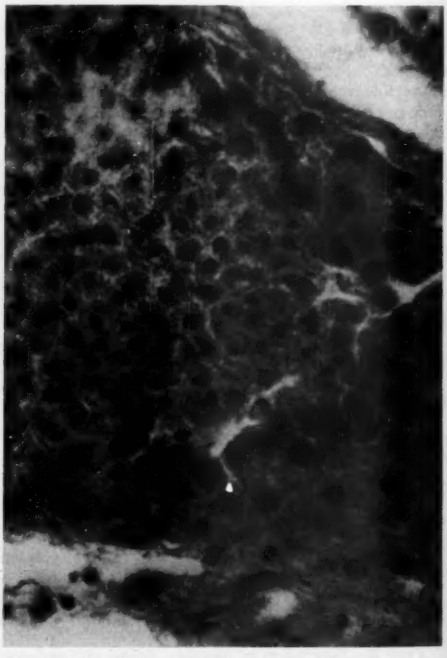


Fig. 2—Seminiferous tubule of an immature bovine testicle. H & E stain; x 380.

The spermatogenic cells basically were arranged in three zones. The large, oval-to-round spermatogonial cells were usually in a single row along the basement membrane. The nuclei of these cells were large, contained much chromatin and, in some cells, showed various stages of cell division.

Toward the lumen was a zone of two to four cell layers which contained the primary and secondary spermatocytes. The primary spermatocytes were most numerous in the area closest to the zone of spermatogonial cells. The primary spermatocyte was usually the larger of the two spermatocyte cell types and was oval to round in shape, with a large, round, darkly staining nucleus occupying most of the cytoplasm of the cell.

The secondary spermatocyte usually was round and had a dark chromatin-prominent nucleus which did not as completely fill the cell as in the primary stage. This secondary group of cells was most numerous in the areas toward the lumen beneath the spermatid cells.

The spermatids were in a zone of variable cell thickness on the spermatocyte layer and toward the lumen of the seminiferous tubule. The spermatids were variable in their structure as they gradually transformed into mature spermia and, at some stages, resembled lymphocytes.

Initially, the spermatids were the cells which formed following division of the secondary spermatocyte. As new daughter cells they were oval to round, had round nuclei, and basically were small editions of the secondary spermatocyte.

As the spermatids transformed into mature spermia, they stayed in close apposition to the sustentacular cells, which nourished or supported these transforming cells as well as some of the newly formed spermia. The transition in the spermatids was one in which the cytoplasm was reduced in amount until it was nearly nonexistent, the nucleus became smaller, the chromatin condensed to become the head of the sperm cell, and a tail developed from the cell to complete the mature sperm cell.

The immature testicle had the same basic structure as the mature. There were several major differences, all of which were related to the inactivity or the immaturity of the organ. The epithelium of the seminiferous tubules was a single layer of cuboidal cells resting on the basement membrane (fig. 2). The lumen of the seminif-

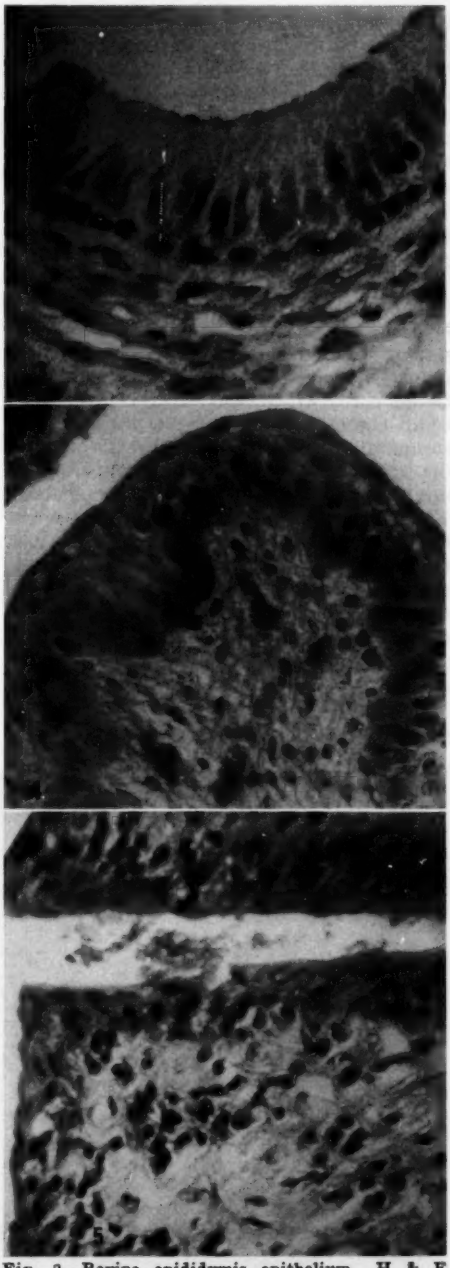


Fig. 3—Bovine epididymis epithelium. H & E stain; x 290.

Fig. 4—Epithelium of the pelvic urethra of a mature bull. H & E stain; x 290.

Fig. 5—Epithelium of urethra in the urethral process of a mature bull. H & E stain; x 430.

erous tubules was not noticeable; this was a striking difference from the spermatozoa-filled lumina of the seminiferous tubules of the mature bull. The interstitial cell groups of the immature testicle were not prominent though, in some areas, a suggestion of their future presence was detectable in groups of fibroblast-like cells with more vesiculate nuclei and more abundant cytoplasm.

THE DUCTULI EFFERENTES

The products of the seminiferous tubules left each lobule by a short duct called the straight tubule (tubule rectus). These ducts converged at the fibrous connective tissue core, which was the origin of the small connective tissue septa. Within this connective tissue, the ducts became irregular in shape; near the head of the epididymis the ductuli efferentes, a series of about 12 ducts, emerged from the connective tissue core at the proximal end of the testicle. At this point, the ductuli efferentes coalesced to form a single duct which was the beginning of the epididymis. The epithelium of the duct changed somewhat, as did the wall of the duct. As the transition occurred from the seminiferous tubule to the straight tubule, the spermatogenic cells disappeared. This left the tall sustentacular (Sertoli) cell as the predominant cell in this area. In the connective tissue septum, the epithelium varied from squamous to cuboidal in the different portions of the rete testis. This variable epithelium continued into the ductuli efferentes.

THE EPIDIDYMIS

At the head of the epididymis, the epithelium was primarily simple columnar to pseudostratified columnar, with the cells showing cilia on the free borders (fig. 3). The nuclei of the columnar cells were long and oval, and all rested at nearly the same level in adjacent cells. Beneath the columnar cells was a zone of polyhedral-to-round cells, with oval-to-round nuclei, that rested on approximately the same level with each other. This layer of cells appeared to be modified connective tissue, and the cells rested on a basement membrane which, in turn, rested on the fibrous connective tissue supporting wall of the ducts. Smooth muscle cells were present in a layer which encircled the wall of the duct. The tortuous bends of the epididymal ducts were held together by light, vascular connective tissue.

The structure of the duct wall in the body of the epididymis was similar to that described for the head of the epididymis,

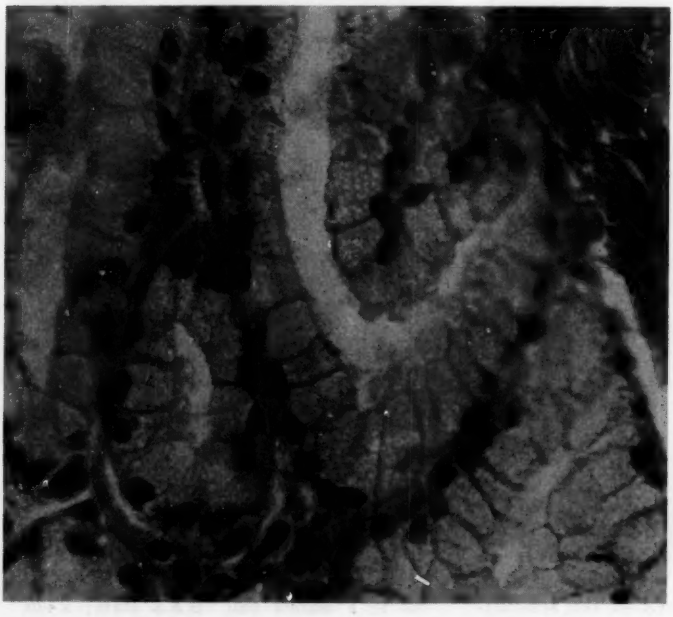


Fig. 6—Mature bovine prostate gland, disseminated part. H & E stain; x 430.

except that the smooth muscle cell layers which encircled the duct had increased in number.

The structure of the duct wall in the tail of the epididymis was similar to that in the head and the body, except that the lumen of the duct was larger, and the circular layer of smooth muscle was a more prominent feature. The connective tissue, consolidating the epididymis into a single mass, was vascular and contained numerous nerve elements.

The Ductus Deferens

The basic structure of the ductus deferens was similar to that of the duct wall in the epididymis. In the ductus deferens, the columnar epithelial cells were somewhat lower, and cilia, though present, were less prominent. The cells beneath the columnar layer were in several layers, causing a protrusion of the columnar epithelium into the lumen. The ductus deferens had a much thicker wall than the epididymis and contained well-developed smooth muscle layers. These muscle layers could be distinguished as an inner longitudinal, a middle circular, and an outer longitudinal layer. The outer longitudinal layer blended with the supporting outer connective tissue of the duct.

The terminal 2 or 3 inches of the ductus deferens was modified into an ampulla. This modification resulted in an increase in the external diameter with little increase in the size of the lumen. The wall of the ductus deferens in this area had become very thick as a result of many irregular folds of the epithelium. The folds contained connective tissue centers and were covered by clear, rather low, columnar cells. The resulting pockets, which basically were evaginations of the epithelium, often branched and anastomosed with each other. The smooth muscle layers were still present but were less distinct. Portions of the epithelial evaginations were found among the muscular layers and gave the appearance of glands.

The Urethra

The epithelium of the pelvic urethra (fig. 4) was transitional. The cells of the superficial layer were flat, those of the middle layer were polyhedral, and those of the basal layer were low columnar or cuboidal. The epithelium rested on a fairly loose vascular connective tissue. The venules in this area were large, thin walled, and represented a form of erectile tissue. Smooth muscle fibers, continuous with those of the disseminated part of the prostate gland, were present in the outer part of the connective tissue layer. The striated urethral muscle surrounded both the urethra and the prostate gland in this area.

The epithelium was transitional at the sigmoid flexure but usually appeared to be thicker than that of the pelvic urethra, as the epithelium was relaxed and thrown up into prominent longitudinal folds with connective tissue centers. The tissue surrounding the urethra was quite vascular, forming cavernous erectile tissue. The connective tissue in the area was very fibrous. The area closest to the urethral epithelium was composed mostly of small, cavernous, venous spaces. Farther out, and continuing to the heavy fibrous connective tissue layer of the shaft of the penis, the venous spaces became larger cavernous sinus-like structures.

In the urethral process, the epithelium (fig. 5) was transitional, thick, and thrown into longitudinal folds; in mature animals, it contained many lymphocytes. The erectile tissue surrounded the urethra in a definite, but poorly developed, layer. Nerve

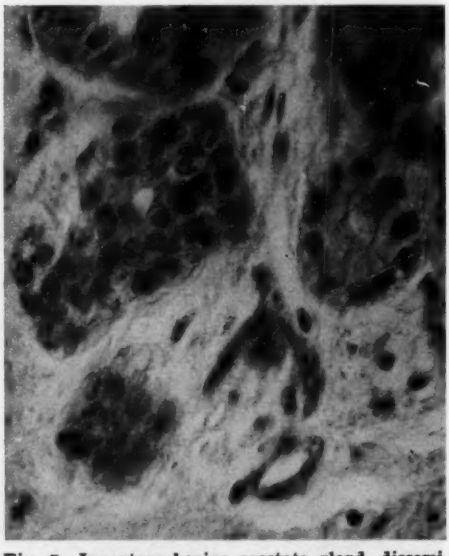


Fig. 7—Immature bovine prostate gland, disseminated part. H & E stain; x 345.

XUM

fibers in the erectile tissue and in the connective tissue were characteristic of this area. The epithelium changed to stratified squamous at the terminal end of the urethral process and was continuous with the epithelium that covered the glans and lined the prepuce.

THE PROSTATE GLAND

The prostate gland was composed of many tubuloalveolar glands that emptied by numerous prostatic ducts into the dorsal wall of the lumen of the pelvic urethra. The secreting portions of the gland were round, oval, or irregular in shape, as seen in the sections. Folds or papillae of epithelium, with central cores of connective tissue, were found projecting into the lumen of the secreting parts of the gland. Portions of the folds occasionally appeared free in the cavities of the secreting portion.

The glandular epithelium was high columnar in the active glands of the mature animal (fig. 6) and low columnar in the immature gland of the 1-day-old calf and in the castrated male (fig. 7). The nuclei of the glandular epithelium were all at approximately the same level in the basal one third of the active cells and in the basal half of the immature or inactive cells.

In the active cells, the cytoplasm exhibited numerous eosinophilic secretory granules. Groups or masses of these granules were also occasionally observed on the free surface of the cells. When secretory granules were numerous and the cytoplasm was full, the nuclei were pushed toward the base of the cell and became slightly flattened.

The epithelial cells lay upon a layer of connective tissue which contained capillaries and elastic fibers. The connective tissue varied, with a seemingly greater amount in immature or inactive glands and less in mature active glands. No basement membrane was apparent.

The striated urethral muscle surrounded the disseminated portion of the prostate. The muscular septa extended in toward the urethra through the prostate gland. Many nerve fibers and some small arteries and veins were present in the connective tissue on the dorsal and lateral surface of the urethral muscle and, in the dorsomedial area, in the muscle itself.

THE SEMINAL VESICLES

In the seminal vesicles, the lumina of the glands were not distinctly outlined because the mucous membrane was folded, producing outpocketing and irregular spaces which anastomosed. The epithelium was

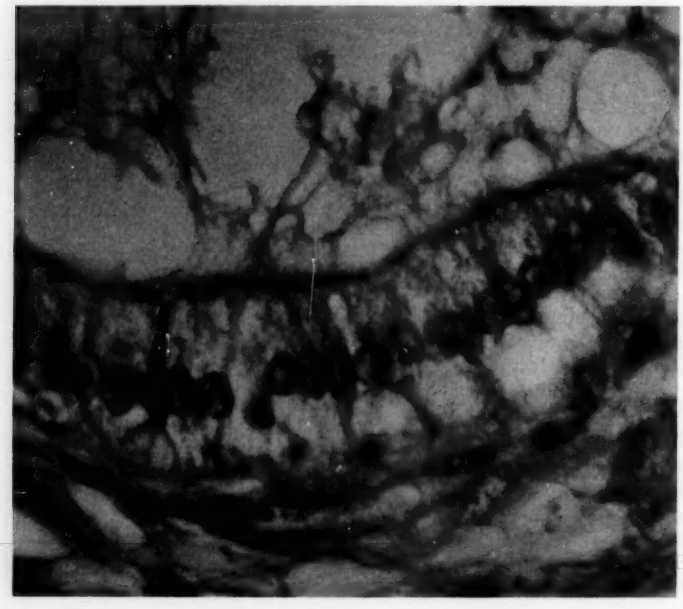


Fig. 8—Seminal vesicle of a mature bull. H & E stain; x 430.

primarily high columnar cells which appeared pseudostratified in the mature active gland (fig. 8). In inactive glands, and in glands from castrated animals, the glandular epithelial cells were low columnar to cuboidal in shape. The nuclei rested in a fairly even row in the basal half of the active cells. A basement membrane was not found; the epithelium rested on a layer of connective tissue containing elastic fibers and capillaries and, in its deeper part, on smooth muscle fibers. The outer surface of the gland was confined by a layer of connective tissue and smooth muscle from which septa extended into the submucosal areas of the gland itself.

The secretion in an active gland appeared as a lightly eosinophilic mass, partially or completely filling the lumen of the secretory areas. The secretory mass was not homogenous but exhibited many irregular, densely packed globules. When this mass was removed, the secretory globules were seen in the distal portions of the secreting cells or on their surfaces. Occasionally, basophilic, calcified concretions were observed in the lumina of the secretory portion of the glands of mature animals.

As the ducts from the secreting areas joined to form larger ducts, the epithelium became lower, until the primary duct was lined by stratified epithelium.

The Bulbourethral Glands

The bulbourethral glands basically were of the tubuloalveolar type. The secreting portions were oval or round to irregular in shape and were arranged in small lobules. The lobules were organized into lobes and the lobes into the complete gland.

The epithelial cells basically were columnar and, in an active gland, the nuclei were in the basal portions of the cells (fig. 9). In immature animals, or in the inactive glands of castrated animals, the cells were low columnar to cuboidal in shape, and the lumina of the secretory areas of the gland were collapsed or reduced in size. The cytoplasm of tall active cells was filled by many mucoid-like droplets. If the secretion was retained in the lumen of an alveolus, the columnar cells appeared somewhat compressed or flattened, due to pressure of the secretion.

No basement membrane was observed, and the epithelium rested on a layer of connective tissue that was similar in structure to that of the seminal vesicles. This subepithelial layer contained elastic fibers, capillaries, and a variable amount of muscle. The surrounding muscle was the striated urethral muscle. The connective tissue septa of the gland proper contained smooth muscle fibers. The capsule surrounding each gland was fibrous connective tissue, from which septa extended into the gland to form the supporting structure.

The duct system developed from a junction of the lumina of the tubular secreting portions. The columnar secreting cells, initially high, became shorter and fewer, but the number of cell layers increased as the ducts approached the urethra. The epithelium became stratified just before the ducts joined the urethra.

The Penis

The penis was a strong, fibrous, inelastic organ. The crura consisted of heavy fibrous connective tissue cylinders which contained erectile tissue and were covered by the striated ischiocavernosus muscle. The fibrous

Fig. 9—Bulbourethral gland of a mature bull. H & E stain; x 380.

XUM

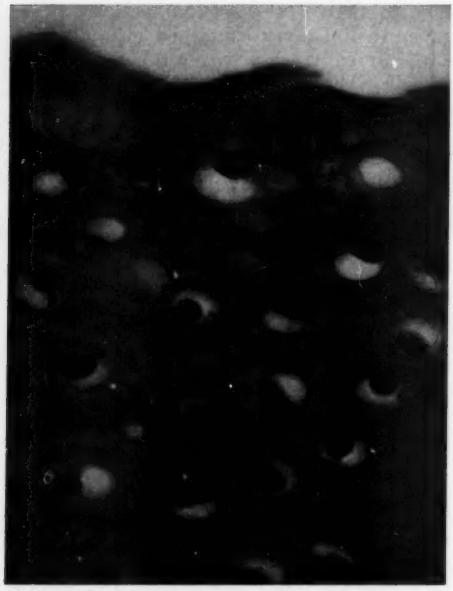


Fig. 10—Epithelium of the glans penis of a mature bull. H & E stain; x 430.

connective tissue layer was attached to the periosteum of the ischium. The erectile tissue of the crura was less fibrous than that of the body of the penis with which it was continuous.

Fig. 11—Epithelium of the prepuce of a mature bull. H & E stain; x 345.

The basic supporting structure of the penis consisted of an outer layer of heavy fibrous connective tissue, called the tunica albuginea. This tunic enclosed the corpus cavernosum penis, the corpus cavernosum urethra, and the urethra. The corpus cavernosum penis was basically erectile tissue that had little erectile ability. The cavernous venous spaces of the erectile tissue were separated by septa which contained much fibrous tissue and had little elasticity.

Two well-developed arteries ran the length of the corpus cavernosum penis, in the area dorsolateral to the urethra. These arteries were continuations of the deep arteries of the penis. Though the blood supply was good, the erectile property of the corpus cavernosum penis was poor, due to the large amount of fibrous connective tissue in the septa between the cavernous spaces.

In the region of the glans, the erectile tissue of the penis gradually was reduced in amount, smooth muscle appeared, the connective tissue septa from the tunica albuginea became more prominent, and the fibrous anterior three to four inches of the organ tapered to a point. The fibrous anterior part was covered by the poorly defined glans penis.

The glans penis consisted of loose connective tissue, several branches of the dorsal arteries of the penis which passed into the area, a few large veins, and numerous nerves and nerve endings. Histologically, there was little typical erectile tissue present.

Beneath the tip of the body of the penis, the urethral process lay attached longitudinally to the glans penis. This process contained the terminal part of the urethra and a small amount of the corpus cavernosum urethra. The glans penis and urethral process were covered by stratified squamous epithelium that was continuous with that of the prepuce (fig. 10).

A gross observation by McLeod,[29] who reported that the urethral groove was converted into an urethral canal by fibrous connective tissue extensions of the tunica albuginea of the penis, was confirmed by the microscopic examinations made in this study. This canal limited the expansion of the corpus cavernosum urethra and the lumen of the urethra.

The Prepuce

The epithelial lining of the prepuce was stratified squamous, hairless, and had a lightly cornified superficial layer (fig. 11). The epithelium was several cell layers thicker than that of the neighboring skin. The subepithelial tissue was a loose areolar connective tissue that gave the prepuce great flexibility.

There was an abrupt change in the type of epithelium at the preputial-skin junction and at the anterior urethral orifice on the urethral process. In the first area, the stratified squamous epithelium of the prepuce joined with the stratified squamous epithelium of the skin, where hair and sebaceous glands were added.

In the second area, the stratified squamous epithelium over the glans met the transitional epithelium of the urethra at the urethral orifice. In contrast, the change was gradual as the preputial epithelium was reflected over the glans, but the epithelium was slightly thicker over the glans proper. The subepithelial tissue was denser, and the epithelium more firmly attached as the apex of the glans was approached. McLeod [29] stated that when erection occurred, the prepuce was laid over the shaft of the penis, thus protecting the penis with the stratified squamous epithelium of the prepuce.

The Scrotum

The wall of the scrotum consisted of a thin layer of skin on the outside, a layer of smooth muscle cells, a layer of fascia, and the tunica vaginalis communis, which comprises the serous inner layer.

The Muscles

The cremaster externus muscle was a band of striated muscle that attached to the tunica vaginalis communis over the testicle. The cremaster internus muscle consisted of a group of indistinct smooth muscle fibers within the structure of the spermatic cord.

The bulbocavernosus muscle was striated muscle that surrounded the bulbous part of the extrapelvic urethra at the ischial arch.

The retractor penis muscle was composed of nonstriated muscle. It began at the first coccygeal vertebra and inserted on the ventral surface of the body of the penis anterior to the sigmoid flexure.

The protractor prepuce muscle was striated muscle that began in the abdominal fascia near the umbilicus and attached to the preputial fascia near the preputial orifice.

The retractor prepuce muscle was a striated muscle that began in the abdominal fascia near the external inguinal ring and inserted in the preputial fascia behind the insertion of the protractor prepuce muscle.

The ischiocavernosus muscle was a striated muscle that covered the crus of the penis near the ischial arch.

Although the muscles were described singly in the preceding, all were paired.

Summary

The majority of previous assumptions regarding the histology of the genital system of the bovine male were found to be correct. The following points, which were not previously reported, or could not be assumed, were observed:

1) The urethra, surrounded by fibrous connective tissue, lay in an enclosed canal rather than in a groove.

2) Microscopically, the fibrous connective tissue surrounding the urethral canal was found to be continuous with the connective tissue of the tunica albuginea of the penis.

3) Microscopically, the accessory genital organs of a castrated male more closely resembled the organs of an immature calf than the organs of a mature bull.

4) The erectile ability of a bull's penis was found to be limited not only by the heavy fibrous tissue tunica albuginea, but also by extensive fibrous tissue septa within the erectile tissue.

5) The epithelium of the prepuce and glans penis were similar in structure, but varied slightly in the thickness and in the density of the subepithelial attachment.

6) Lymphocytes in the epithelium of the anterior part of the urethra were not uncommon in the mature bull.

References

[1] Asdell, S. A.: Cattle Fertility and Sterility. Little, Brown & Co., Boston, 1955.

[2] Bascom, K. F.: The Interstitial Cells of the Gonads of Cattle with Special Reference to Their Embryonic Development and Significance. Am. J. Anat., *31*, (1923): 223.

[3] Bissonnette, I. H.: The Development of the Reproductive Ducts and Canals in the Freemartin with Comparison of the Normal. Am. J. Anat., *33*, (1924): 1924.

[4] Blom, E.: Interpretation of Spermatic Cytology in Bulls. Fert. & Ster., *1*, (1950): 223.

XUM

[5] Cowdry, E. V.: A Textbook of Histology. Lea & Febiger, Philadelphia, 1938.

[6] Cupps, P. T., Laben, R. C., and Mead, S. W.: The Histology of the Pituitary, Testis and Adrenal in Relation to Reproduction in the Bull. J. Dai. Sci., *37*, (1954): 1074–1087.

[7] Deakin, A.: Field Fertility Tests and Causes of Sterility of Bulls. Exptl. Farms Serv., Dept. of Agric., Canada, 1943 (mimeo).

[8] Deysack, L. J.: The Comparative Morphology of the Erectile Tissue of the Penis with Special Emphasis on the Probable Mechanism of Erection. Am. J. Anat., *64*, (1939): 111–131.

[9] Easley, G. T., Leonard, R. H., and Trotter, D. M.: Bacteriological, Pathological and Clinical Studies of the Reproductive Tract of the Hereford Cow and a Bacteriological Study of Hereford Bull Semen. North Am. Vet., *32*, (1951): 258–266.

[10] Engle, E. T.: Studies on Testis, Ovary, Egg and Sperm. Charles C. Thomas, Publisher, Springfield, Ill., 1954.

[11] Fossland, R. G.: Histological Development in the Postnatal Bovine Testis. J. Dai. Sci., *37*, (1954): 669.

[12] Foust, H. L., and Getty, R.: Veterinary Histology and Embryology, Burgess Publishing Co., Minneapolis, 1955.

[13] Gasner, F. X.: Reproductive Problems in Bulls. In "Reproduction and Fertility—A Symposium." Michigan State Univ. Press, East Lansing (1955): 87–98.

[14] Greep, R. O.: Histology. Blakiston Co., Philadelphia, 1953.

[15] Ham, A. W.: Histology. J. B. Lippincott Co., Philadelphia, 1953.

[16] Hancock, J. L.: The Spermatozoa of Sterile Bulls. J. Exptl. Biol., *30*, (1953): 50–56.

[17] Haq, I.: Some Morphological Characteristics of the Epithelial Cells Lining the Genital Tract of the Bull. Vet. J., London, *104*, (1948): 5–22.

[18] Harrison, R. G.: The Comparative Anatomy of the Blood Supply of the Mammalian Testis. Proc. Zool. Soc. London, *119*, (1949): 325–344.

[19] Herman, E. A., and Madden, F. W.: Artificial Insemination of Cattle. Lucas Bros., Inc., Columbia, 1947.

[20] Hill, H. J., and Gasner, F. X.: Effect on Quality of Semen. I. Testicular Biopsy in the Bull. Fert. & Ster., *6*, (1955): 215–227.

[21] Hill, H. J., and Gasner, F. X.: Effect on Semen. II. Testicular Biopsy in the Bull. Fert. & Ster., *6*, (1955): 290–301.

[22] Hooker, C. W.: The Postnatal History and Function of the Interstitial Cells of the Testis of the Bull. Am. J. Anat., *74*, (1944): 1–37.

[23] Kimball, A., Twiehaus, M. J., and Frank, E. R.: *Actinomyces Bovis* Isolated from Six Cases of Bovine Orchitis—A Preliminary Report. Am. J. Vet Res., *15*, (Oct., 1954): 551–553.

[24] Laing, J. A.: Fertility and Infertility in the Domestic Animals. Baillière, Tindall and Cox, London, 1955.

[25] Lillie, F. R.: The Free-martin—A Study of the Actions of Sex Hormones in Foetal Life of Cattle. J. Exptl. Zool., *23*, (1917): 371.

[26] Macklin, C. C., and Macklin, M. T.: The Seminal Vesicles, Prostate, and Bulbourethral Glands. In "Special Cytology—Cowdry." Paul B. Hoeber, New York, N.Y., 3, (1932): 1773.

[27] Marsh, H., and Safford, J. W.: The Effect of Deferred Castration on Urethral Development in Calves. J.A.V.M.A., *130*, (April 15, 1957): 342–344.

[28] Maximow, A. A., and Bloom, W.: A Textbook of Histology. Williams & Wilkins Co., Baltimore, 1940.

[29] McLeod, W. M.: Bovine Anatomy. 2nd ed. Burgess Publ. Co., Minneapolis, 1958.

[30] Melampy, R. M., Cavozos, L. F., and Duncan, G. W.: The Composition and Histochemistry of Bull, Ram, Boar, and Rooster Testes. In "Reproduction and Infertility—A Symposium." Michigan State Univ. Press, East Lansing (1955): 34–44.

[31] Miller, F. W., and Everrette, T. E.: Technique for Obtaining Spermatozoa for Physiological Dairy Studies and Artificial Insemination. J. Agric. Res., *48*, (1934): 941–947.

[32] Milne, F. H.: Penile and Preputial Problems in the Bull. J.A.V.M.A., *124*, (1954): 6–11.

[33] Perkins, J. R., Olds, D., and Seath, D. M.: A Study of 1,000 Bovine Genitalia. J. Dai. Sci., *37*, (1954): 1158–1163.

[34] Perry, E. J.: The Artificial Insemination of Farm Animals. Rutgers Univ. Press, New Brunswick, 1945.

[35] Phillips, R. W., and Andrew, F. N.: The Development of the Testes and Scrotum of the Ram, Bull, and Boar. Massachusetts Agric. Exper. Sta. Bull. 331, (1936): 16.

[36] Rollinson, D. G. L.: Mullerian Duct Remnants in the Bull. Vet. Rec., *62*, (1950): 107–108.

[37] Santamarina, E., and Reece, R. P.: Normal Development of the Germinal Epithelium and Seminiferous Tubules of the Bull. Am. J. Vet. Res., *18*, (April, 1957): 261–278.

[38] Smith, P. E.: Bailey's Textbook of Histology. Williams & Wilkins Co., Baltimore, 1940.

[39] Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. W. B. Saunders Co., Philadelphia, 1953.

[40] Trautman, A., and Febiger, J.: Fundamentals of the Histology of Domestic Animals. Comstock Pub. Associates, Ithaca, 1952.

[41] Williams, W. L.: The Diseases of the Genital Organs of Domestic Animals. George Banta Publ. Co., Menasha, Wisc., 1943.

[42] Wodsedalek, J. L.: Studies on the Cells of Cattle, with Special Reference to Spermatogenesis, Oogonia and Sex-Determination. Biol. Bull. 38, (1920): 290.

Scientific Notes

Conference on Histopathology of Experimental Avian Lymphomatosis

A workshop conference was held at the U.S. Regional Poultry Research Laboratory, East Lansing, Mich., on March 17–19, 1958, to study pathological material and to express opinions regarding the principal morphological changes that take place following inoculations with cell-free preparations of strain RPL 12 and other sources of virus. Pathologists, hematologists, and virologists familiar with the natural, as well as the experimental, neoplasia of chickens, examined a series of specimens from representative cases in which one or the other of the two principal manifestations existed.

The majority of the group agreed to the following:

1) The predominant cell of the "intravascular" neoplasm induced by cell-free inoculum of strain RPL 12 is a very young erythroblast (proerythroblast).

2) The predominant cell of the "extravascular" neoplasm called visceral lymphomatosis induced by cell-free inoculum of strain RPL 12 is a very immature cell which, on the basis of comparative studies, is considered to belong to the lymphocytic series.

3) The naturally occurring (spontaneous) cases of visceral lymphomatosis show evidence of a graded spectrum of malignancy as exhibited by the predominating cell in individual cases. One case of visceral lymphomatosis may exhibit an immature type cell, whereas another may exhibit a relatively mature type cell. Thus, the predominant cell probably belongs to the same developmental series, but at a different state of maturation.

4) As judged by the gross and microscopic character of the lesions, the disease designated as visceral lymphomatosis produced by cell-free material from strains RPL 12, 16, and 23 is similar to naturally occurring visceral lymphomatosis.

5) The disease designated as erythroblastosis is regarded as arising from neoplastic proliferation of the sinusoidal endothelium of the bone marrow.

6) The designation "visceral lymphomatosis" is regarded as a broad term indicating neoplasia of cells of the lymphocytic series which, in individual cases, shows varied degrees of malignancy. In any given case, the degree of the malignancy could be expressed by the use of such terms as lymphoblastomatosis for the tumors of the more immature cell type, or lymphocytomatosis for those of the more differentiated ones.

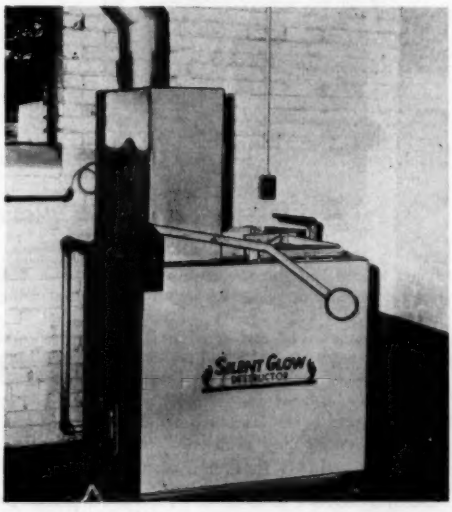
The conferees were: Drs. B. R. Burmester, Alfred M. Lucas, M. Adrian Gross, and Willard G. Walter, Regional Poultry Research Laboratory, Animal Husbandry Division, ARS, U.D.S.A., East Lansing, Mich.; C. le Q. Darcel, Department of Agriculture, Veterinary Research Laboratory, Lethbridge, Alta.; Vittorio Defendi, the Wistar Institute, Philadelphia; Oliver P. Jones, Department of Anatomy, University of Buffalo, Buffalo, N.Y.; Erwin Jungherr, Department of Animal Diseases, University of Connecticut, Storrs; G. S. McKee, Inspection Branch, Poultry Section, Agricultural Marketing Service, U.S.D.A., Washington, D.C.; and Carl Olson, Jr., Department of Veterinary Science, University of Wisconsin, Madison.

Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

Medical Crematory Destructor

A medical crematory destructor for destroying diseased organic matter and other medical wastes has been developed by the Silent Glow Burner Corp.



The operation of this unit is smokeless, odorless, and fly-ash free. The casing is constructed of heavy electrically welded steel, coated with a finish to prevent corrosion. The unit is mounted on heavy steel skids. The interior is of an insulated refractory construction; refractory temperature ratings allow an adequate temperature margin.

The unit uses No. 2 fuel oil and has an automatic ignition, automatic temperature control, and an automatic timer. Controls prevent the opening of the charge chamber door until necessary temperatures or complete destruction have been attained.

XUM

New Magnetic Stirrer

The rigid clamp of a newly designed magnetic stirrer, manufactured by Arthur S. LaPine and Co., is cast integrally with the base so that the stirrer can be mounted on a support rod or used on a bench top with or without the support rod.

A shaded pole electric motor spins the Alnico V permanent bar magnet horizontally about the motor axis and the molded stirring bar in the vessel follows the rotating magnetic field, stirring without any mechanical coupling. Increasing the motor

speed by raising the rheostat setting causes a vortex in the liquid, resulting in an effective stirring action.

The diameter of the housing is 4¼ inches, and the total height is 3½ inches. The clamp accommodates rods of up to ¾-inch diameter. Distance from the center of the support rod to the center of the stirrer is the standard 4½ inches. Any nonmagnetic vessel may be used.

Coordinate Plate and Film Comparator

A new optical measuring instrument (Coordinate Plate and Film Comparator, developed by Gaertner Scientific Corp., has been designed for precise coordinate measurements on plates and films and is especially useful for ultracentrifuge and electrophoresis plates and films.

The instrument reads to 0.002 mm., has a standard magnification of × 15, and a measuring range of 50 mm. by 100 mm. Magnification can be changed by the use of interchangeable objectives. Larger size plates or films can be accommodated by accessory plate holders.

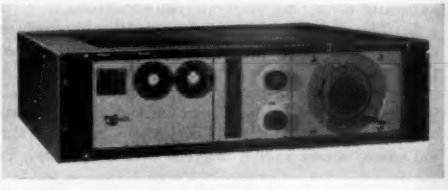
Instead of the conventional eyepiece, an erecting projection head displays the image on an 11-cm. projection screen. The instrument has full

50-mm. precision measuring screws and a stage rotation of ±5 degrees for plate alignment. The high-intensity substage illuminator, collimator, fresnel lens, and light shield make it possible to view the image under ordinary room lighting conditions.

The instrument is available with a square coordinate stage or with a round 360-degree rotary stage. The rotary stage reads to one minute of arc and is valuable where angles or polar coordinates must be measured. Both instruments are calibrated either in the inch or in the metric system.

Versatile Radioactivity Counter

A cold cathode glow tube scaler for general radioactivity counting, designed for integral pulse height analysis in scintillation or proportional counting, is being manufactured by Baird-Atomic, Inc.



This new model (B-A Model 131 A), a version of the Dekatron glow tube scaler, has a 7-decade capacity and a built-in timer for variable preset timing. It contains an ultralinear, ultrastable expander amplifier-type pulse height selector, a resolving time of less than 5 microseconds, and an electronic scale of 1,000 followed by a 4-digit electromechanical register. The plug-in construction sub-units are removable from the front.

This model may be used with any compatible preamplifiers and high voltage supplies to form ultrastable counting systems.

XUM

for **MASTITIS** *of*

dry and lactating cows

NEW SQUEEJET[T.M.*]

single-dose dispenser for

FURACIN® SOLUTION *veterinary*

brand of nitrofurazone

- delivers one full dose
- is discarded after using
- saves time
- is convenient, especially when a number of cows are to be treated
- unique package

*design patented

FURACIN has been shown to be strikingly effective in controlling mastitis under field conditions.[1,2] Of 7,123 lactating cows with acute mastitis, fair to excellent results were obtained in 5,597 (78%). Of 3,418 dry cows which had had mastitis during their previous lactating period, fair to excellent results were obtained in 3,104 (90%).

SUPPLIED A water-miscible liquid of FURACIN 0.2% in polyethylene glycol and water. Single-dose SQUEEJET disposable dispenser of 30 cc. Still available, bottles of 500 cc. with rubber stopper and 1 gal.

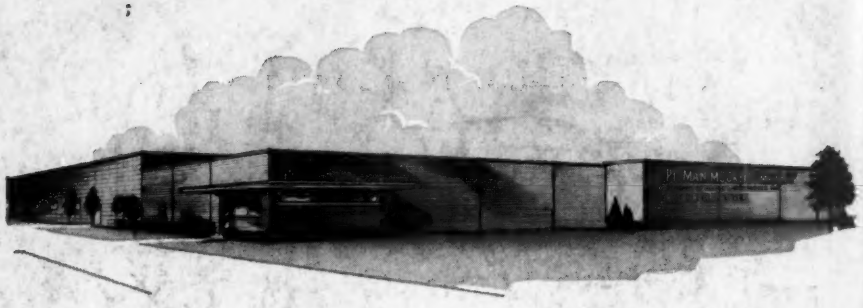
1. Mires, M. H., and Chadwick, R. H.: Vet. News **10**:3 (Jan.-Feb.) 1947. 2. Mires, M. H.: J. Am. Vet. M. Ass. **117**:49 (July) 1950.

All Eaton Veterinary Specialties Are Available Through Your Professional Veterinary Distributor.

NITROFURANS—a *new* class of antimicrobials— neither antibiotics nor sulfonamides

EATON LABORATORIES, NORWICH, NEW YORK

XUM



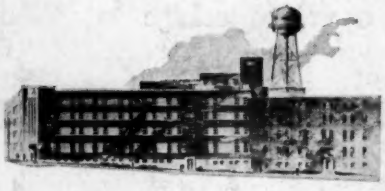
new research facilities for

PITMAN-MOORE COMPANY

Division of Allied Laboratories, Inc.

A new center for research in both human and veterinary medicine is under construction by Pitman-Moore Company at a cost of approximately $1,500,000. When completed, early in 1959, this new building will add 41,000 square feet of space for laboratories, offices, and technical equipment in the company's Research Center near Zionsville, Indiana.

The Indianapolis laboratories, where pharmaceuticals are produced.



Within this organization originated the policy: Sales to Graduate Veterinarians ONLY

The Zionsville laboratories, where biologicals are produced.

PITMAN-MOORE COMPANY, DIVISION OF
ALLIED LABORATORIES, INC.
INDIANAPOLIS 6, INDIANA

XUM